



Comparison of Transcript Profiles in Wild-Type and *o2* Maize Endosperm in Different Genetic Backgrounds

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Abstract

Mutations in the *Opaque2* (*O2*) gene of maize (*Zea mays* L.) improve the nutritional value of maize by reducing the level of zeins in the kernel. The phenotype of *o2* grain is controlled by many modifier genes and is therefore strongly dependent on genetic background. We propose two hypotheses to explain differences in phenotypic severity in different genetic backgrounds: (i) Specific genes are differentially (*o2* vs. wild-type) expressed only in certain genotypes, and (ii) A set of genes are differentially expressed in all backgrounds, but the degree of differential expression differs in different backgrounds. To determine the extent to which these two hypotheses contribute to determining the severity of *o2* in different genetic backgrounds, we identified transcripts likely to be differentially expressed in several genetic backgrounds by transcript profile comparison of endosperm RNA pools from eight *o2* inbred lines and their wild-type counterparts. The inbred line B46 was identified as having severe *o2* phenotypes while the line M14 was identified as having minimal *o2* phenotypes. The degree of wild-type vs. *o2* differential expression of transcripts was determined for these two lines. We found that most genes that are downregulated by *o2* tend to be differentially expressed to a greater degree in B46 than in M14, while upregulated genes tend to be more highly differentially expressed in one genetic background or the other. Thus, hypothesis one functions more prominently for upregulated genes while hypothesis two functions most prominently for downregulated genes.

THE MAIZE (*Zea mays* L.) *opaque2* (*o2*) mutation causes mutant kernels to transmit less light than their wild-type counterparts. With the discovery that this mutation also conferred elevated levels of lysine and other essential amino acids (Mertz et al., 1964) and therefore increased nutritional quality to mutant kernels, this mutation became the subject of intense research. The increased levels of essential amino acids characteristic of the *o2* mutation are accompanied by reduced levels of the major seed storage proteins in the maize kernel, the zeins (Mertz et al., 1964). Unfortunately, the *o2* mutation was not put to immediate use for development of maize varieties with improved nutrition because of the many pleiotropic effects of this mutation that reduce its agronomic adaptability. For example, *o2* kernels are more susceptible to cracking than wild-type kernels (Lambert et al., 1969). In addition, poor agronomic traits including seedling emergence, field establishment, grain yield and susceptibility to ear

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Abbreviations: *o2*, *opaque2*; PPK, orthophosphate dikinase; G3PD, Glyceraldehyde-3-phosphate dehydrogenase; EF1 α , Elongation factor 1 alpha; B32, ribosome inactivating protein; LKE/SDH, Lysine-ketoglutarate reductase/saccharopine dehydrogenase; PPDE, Posterior probability of differential expression.

and kernel rots (Nass and Crane, 1970; Loesch et al., 1976; Loesch et al., 1978) are associated with the *o2* mutation and have discouraged utilization of the *o2* mutation despite its nutritional advantages.

There are many examples of genetic background influencing *o2* phenotypes. For example, kernel shearing force was determined for a series of inbred lines and their *o2* conversions and the severity of the phenotype was found to be dependent on the genetic background (Loesch et al., 1977). In addition, transcript levels of zein genes and zein protein levels in *o2* endosperm have been shown to be genetic background-dependent (Bernard et al., 1994; Ciceri et al., 2000), along with seed opacity, which has been correlated with a reduced zein content in several *o2* inbred lines (Schmidt, 1993). Similarly, the phenotypic variation in lysine content of *o2* inbred endosperm is also related to genotype (Paez et al., 1969; Moro et al., 1996; Wang and Larkins, 2001). One explanation for the impact of genetic background on the *o2* phenotype is that epistatic interactions occur between *o2* and alleles specific to a given genetic background. It has been shown that the phenotype of *o2* is controlled in part by 'modifier genes' (Paez et al., 1969; Wessel-Beaver, 1982) which confer quantitative genetic inheritance on many of the detrimental seed traits (Wessel-Beaver et al., 1985). Thus, it is possible to select *o2* genotypes with reduced opacity and therefore better agronomic characteristics (Lambert and Chung, 1995). Researchers at the International Maize and Wheat Improvement Center (CIMMYT) in Mexico used recurrent selection for endosperm hardness in *o2* germplasm to develop Quality Protein Maize (QPM) (Vasal et al., 1980) which is characterized by an elevated lysine content but lacking the detrimental phenotypes (Bjarnason and Vasal, 1992; Gevers and Lake, 1992). Maize breeders are currently using QPM germplasm to develop nutritionally improved varieties.

Molecular characterization of the *O2* gene has greatly contributed to our understanding of the molecular and biochemical mechanisms leading to the many phenotypic effects of the *o2* mutation. The *O2* gene encodes a basic leucine zipper transcriptional activator (Hartings et al., 1989; Schmidt et al., 1990) which binds the promoter region of 22 kDa α -zein maize storage protein genes (Schmidt et al., 1992; Ueda et al., 1992) and the β -prolamin gene promoters (So and Larkins, 1991; Cord Neto et al., 1995). The identification of these interactions explains the transcriptional reduction of the 22 kDa α -zein genes and 14 kDa β -zein genes in *o2* mutants.

In addition to its involvement in regulating seed storage protein synthesis, the *O2* protein has been found to directly interact with the promoter region of

several genes including the gene encoding cytoplasmic pyruvate orthophosphate dikinase (*cyPPDK1*), an enzyme active early in seed endosperm development (Maddaloni et al., 1996), a gene encoding lysine-ketoglutarate reductase/saccharopine dehydrogenase (LKR/SDH), a bifunctional enzyme involved in lysine degradation (Kemper et al., 1999), and with palindromic sequences in the promoter of the *b-32* ribosome-inactivating protein gene (Lohmer et al., 1991; Bass et al., 1992). The sensitivity to lysine of a lysine insensitive mutant of aspartate kinase is reduced in combination with *o2*, suggesting that this gene may be regulated by *O2* as well (Brennecke et al., 1996; Wang et al., 2001). The large number of genes affected by the *o2* mutation was demonstrated using transcriptional profiling with Affymetrix (Santa Clara, CA) Gene Chips. In this study, 126 transcripts were up- or downregulated more than three fold by *o2* in the W64A genetic background (Hunter et al., 2002). A study of proteins that accumulate differentially in *o2* and wild-type endosperm from seven genetic backgrounds identified 36 proteins that were accumulated differentially in all seven backgrounds (Damerval and LeGuilloux, 1998). It is not clear how the genes altered by the *o2* mutation condition the major phenotypes of the mutation, but the diversity of these genes is certainly consistent with the large variety of pleiotropic effects caused by *o2*.

Transcripts that are differentially expressed only in certain genetic backgrounds create a problem for characterizing the *o2* mutant because differential expression of these transcripts is not a strictly required component of the *o2* phenotype. Transcripts that are differentially expressed in all genetic backgrounds may be necessary components of the *o2* phenotype and therefore are potentially more informative than transcripts that are differentially expressed only in a few genetic backgrounds. One objective of this study is to identify a set of transcripts likely to be differentially expressed in most genetic backgrounds. We reasoned that averaging differential expression data across genotypes would minimize the signal of transcripts that were differentially expressed in only a few genotypes. To do this, we used pooled transcripts from eight inbred line pairs, giving a genotype-averaged set of transcripts that were likely to be responsive to *o2* in many genetic backgrounds. By averaging out the signals of transcripts that were differentially expressed in only a few genetic backgrounds, it was possible to reduce the number of transcripts requiring characterization to understand the molecular basis of *o2* phenotypes.

Given that so much of the effort in breeding programs is devoted to minimizing the detrimental phenotypes of the *o2* mutation, better understanding of the genetic control of these phenotypes would be beneficial. We propose two hypotheses to explain differences in phenotypic severities found in dif-

ferent genetic backgrounds. First, differences in phenotypic severity could be due to differences in the degree to which transcript levels change due to mutation of *O2*. These changes would occur within a set of transcripts that are differentially expressed in most lines. Second, these differences may be due to specific transcripts whose levels are differentially changed by mutation of *O2* in some genetic backgrounds and not in others. A second objective of this study is to determine the extent to which each of these two proposed mechanisms function to control phenotypic severity by comparing the differentially expressed transcripts identified in a genetic background with severe *o2* effects to those identified in a genetic background with minimal *o2* effects.

Results

Identification of Transcripts Differentially Expressed in Pooled Samples

Transcripts that are differentially expressed only in certain genetic backgrounds make it difficult to determine how a molecular lesion in *o2* causes the observed phenotypes because its effect is present only in certain genotypes. To overcome this problem, we sought to identify a core set transcripts in which the number of genotype-specific differentially expressed transcripts was minimized. To do this, we performed a genotype-averaged hybridization in which mRNA from immature endosperms of eight wild-type inbred lines (A619, B14A, B45, B46, B57, B66, B73 and M14) was pooled and compared to pooled endosperm mRNA from the *o2* conversions of the same eight inbred lines. Because the degree of differential expression is averaged across eight genotypes, differential expression of transcripts identified in this experiment is more likely to be an obligate component of the *o2* response than differential expression of transcripts identified in a single genotype wild-type vs. *o2* comparison, which could be due to genotype-specific responses. Transcripts identified as differentially expressed in the pooled genotypes hybridization are good candidates to characterize further in efforts to elucidate the mechanism of *o2* function. We denote them “universally *o2* affected” transcripts to underscore the fact that *o2* is likely to have a consistent, strong effect on them in many, if not all, backgrounds.

Among the 37 universally *o2* affected transcripts most likely to be differentially expressed (*P* value < 0.001) in the comparison of genotype pools, twenty-five of these transcripts were upregulated in the mutant and 12 of them were downregulated. Of those with known functions, transcripts likely to be involved in signal transduction were a promi-

nent group, represented by three transcripts with homology to GTP-binding proteins and two with homology to protein kinases. Two transcripts had homology to DNA binding proteins, which was particularly interesting given that *o2* is a transcription factor. Other transcripts that fell in this group had homology to genes encoding ribosomal proteins,

The *opaque2* mutation is used in breeding programs around the world to produce maize varieties with improved nutrition. The poor agronomic quality of *opaque2* grain is a major obstacle in these programs, and a great deal of effort has been required to overcome this obstacle.

S-adenosyl methionine decarboxylase and coumarate CoA ligase. The fact that no zeins were on this list was surprising given that reduction in zein levels is a hallmark of the *o2* mutation. The threshold level for selecting these genes was very stringent, and it is likely that we have excluded from this list some genes that were differently expressed. Also, differential expression of zein transcripts may be genotype dependent. Zeins may be differentially expressed on average in most genetic backgrounds, but it may be different zein transcripts are differentially expressed in each genetic background. If this is the case, then because we pooled different genotypes before measuring differential expression in this experiment, the likelihood of a given zein transcript being differentially expressed would be reduced.

Assessment of Kernel Phenotypes

One of the objectives of this study was to identify molecular differences in different genetic backgrounds with different phenotypic severities. These differences could help to explain differences in *o2* phenotypic severity in these genetic backgrounds. To do this, it was first necessary to determine the severity of *o2* phenotypes in several genetic backgrounds. We ranked the eight wild-type inbred lines and their *o2* conversions that were used in the pooled genotypes microarray study according to the severity of the *o2* mutation on five phenotypes considered to be influenced by *o2*. These phenotypes were lysine content, kernel density, germination rate, seedling shoot-to-root ratio and seed weight.

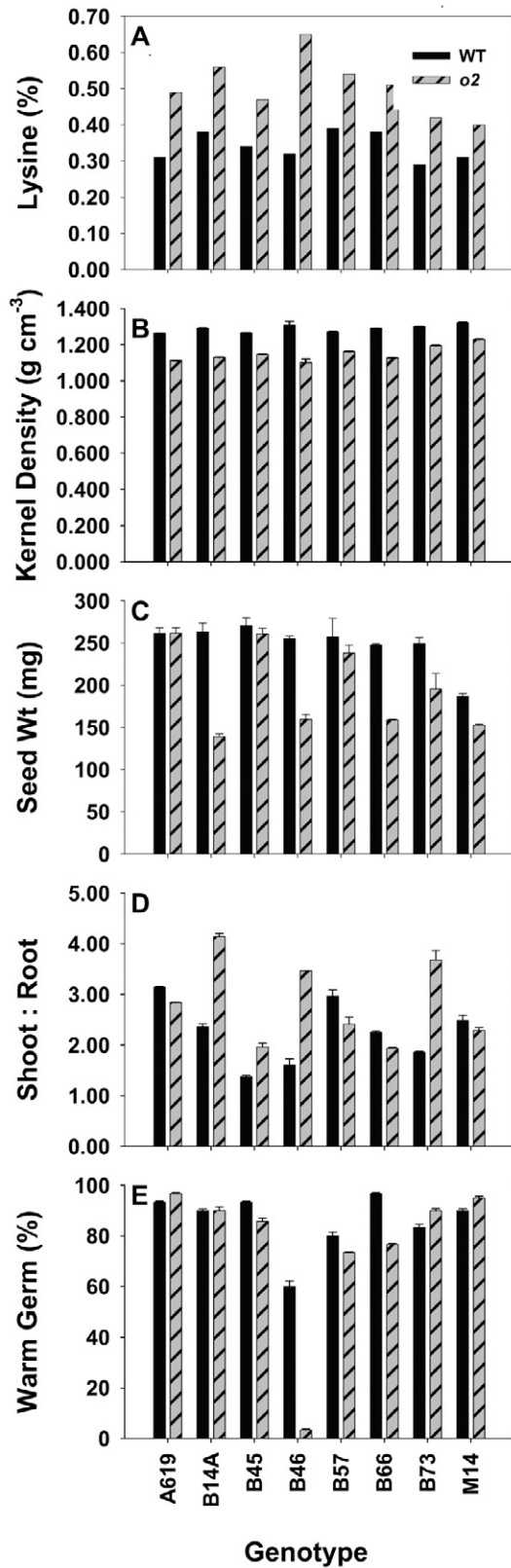


Figure 1. Comparison of kernel phenotypes in wild-type and *o2* versions of eight inbred lines. Error bars indicate one standard deviation from the mean. Error bars are not shown for lysine content because each sample was measured once.

The first phenotype examined was total (free + bound) kernel lysine content. Each of the eight mutants had higher lysine content per tissue mass than their wild-type counterpart (Fig. 1A). Among the eight *o2* mutants, B46*o2* had the highest lysine content (0.65%), which was more than twice the amount in the wild-type B46 (0.32%). M14*o2* had the lowest lysine content among the mutants (0.40%), which was less than a 25% increase in lysine content relative to the wild-type M14 (0.31%). Thus, conversion to *o2* had the greatest impact on B46 and the smallest impact on M14 with regard to lysine content.

Kernel density in the wild-type lines was significantly higher than in their *o2* mutant counterparts, 1.29 versus 1.15 g per cubic centimeter on average, respectively (Fig. 1B). As with lysine content, B46 experienced the most extreme change on conversion to *o2*, while M14 had the least extreme change in kernel density.

Measures of seed weight were generally higher in the wild-type lines relative to their *o2* conversions (Fig. 1C), averaging 249 mg in the wild-type samples and 196 mg in the *o2* samples. The difference between wild-type and *o2* was smallest in the inbred line A619 and largest in the line B14A.

As a reflection of the ability of seedlings to allocate resources efficiently and equitably to meristematic sinks, the ratio of shoot-to-root tissue dry mass of normal seedlings was determined. The ratio was lower on average (2.25 to 2.84, respectively) in the wild-type relative to their *o2* conversions, but four lines did have a higher shoot-to-root ratio for the wild-type version than for the *o2* conversion (Fig. 1D). Warm germination percentages were above 70% with the exception of both B46 lines (Fig. 1E).

The rank of the eight inbred line pairs in each of the five phenotypic evaluations is summarized in Table I. Taken together, these experiments support the classification of B46 as a line with extreme phenotypic effects on conversion to *o2* and M14 as a line with minimal phenotypic effects on conversion to *o2*. We therefore focused on these two lines for the remaining phenotypic analysis.

An important consequence of the *o2* mutation is to reduce the accumulation of certain zein polypeptides. HPLC analysis of alcohol-extractable proteins can be used to quantify individual zein polypeptides. While other methods result in more efficient extraction of some zeins (Wallace et al., 1990), alcohol extraction followed by HPLC analysis provides a precise method for comparative analysis of the levels of individual zeins (Paulis et al., 1991; Wilson, 1991). Examination of the alcohol-extractable proteins from wild-type and *o2* versions of B46 and M14 revealed a reduction in zein levels that was genetic background-dependent. In B46*o2*, all

the zein protein levels were decreased dramatically. In the chromatogram of this mutant, the only peaks that were visible were γ -zein peaks and these were much lower than in the wild-type (Fig. 2 A). Expression of other zeins was reduced so dramatically that none of them could be detected. In contrast, in M14o2, some α -zein levels were reduced significantly, but some were not (Fig. 2 B). The 27 kDa γ -zein did not show a large difference in abundance between wild-type and o2. Interestingly, the level of 16 kDa γ -zein was higher in M14o2 than its wild-type, which is in agreement with a previous study conducted in the W64A genetic background (Kodrzycki et al., 1989; Paulis et al., 1991). The extent of reduction of α -zein levels in the o2 line compared to the wild-type line was much greater in B46 than in the M14 mutant.

Hypotheses to Explain Differences in o2 Phenotypic Severities in Different Genetic Background

Taken together, the experiments above support the classification of B46 as a line with severe phenotypes on conversion to o2, and M14 as a line with mild phenotypes on conversion to o2. We propose two non-mutually exclusive hypotheses to explain the difference in phenotypic severities observed in these two lines. First, it may be that o2 affects the expression of a set of transcripts in both lines, but these transcripts are differentially expressed to different degrees in the two different genetic backgrounds. We call this hypothesis the degree of differential expression hypothesis.

The second hypothesis we propose to explain the difference in phenotypic severities observed in the B46 and M14 genetic backgrounds states that o2 affects the expression of different sets of transcripts in each of these backgrounds, and these genotype-specific transcripts attenuate the severity of the o2 phenotype. We call this hypothesis the gene-specific transcript hypothesis.

These hypotheses should be readily distinguished by global transcript profiling of the two genetic backgrounds that we identified as having severe and mild phenotypic effects on conversion to o2, B46 and M14, respectively. Following transcript profiling, the degree of differential expression of each transcript in B46 can be plotted against the degree of differential expression of each transcript in M14 (Fig. 3). Relative to the universally o2 affected transcripts discussed above and to the two hypotheses proposed, we expect the following from such plots. First, the universally o2 affected transcripts should be those strongly and equally affected across backgrounds, mak-

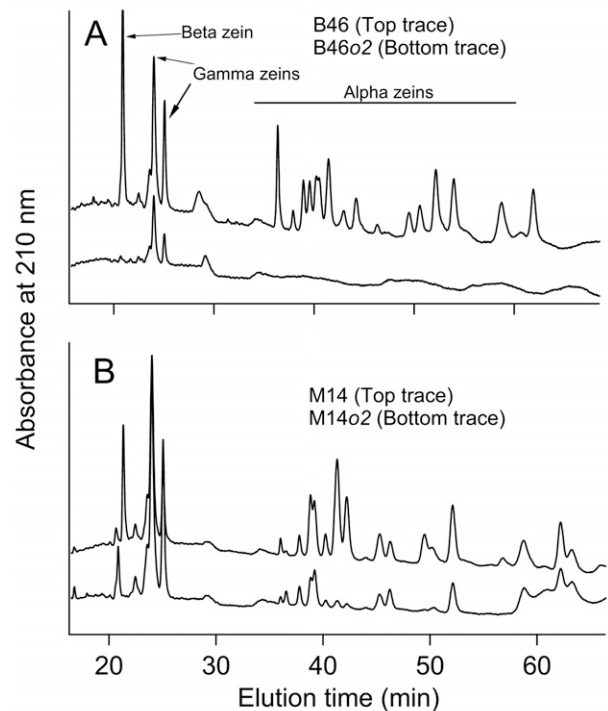


Figure 2. HPLC chromatograms of alcohol-extractable zein proteins from 14 DAP wild-type and o2 mutant endosperm. **A:** Comparison of HPLC chromatograms of B46 wild-type and B46o2 mutant. The protein levels of all four zein families are reduced significantly in B46o2 mutant. **B:** Comparison of HPLC chromatograms of M14 wild-type and M14o2 mutant. The β -zein and some of the α -zeins protein levels are reduced significantly, while the γ -zein levels are increased in M14o2 mutant.

ing them fall close to the 45 degree and 225 degree rays. Transcripts conforming to the degree of differential expression hypothesis should fall close to a ray that is nearer to the B46 axis than 45 degrees for downregulated transcripts, or 225 degrees for upregulated transcripts (Fig. 3A). Finally, transcripts conforming to the gene specific differential expression hypothesis should fall close to the B46 axis itself for those transcripts differentially expressed specifically in B46, and close to the M14 axis for those transcripts differentially expressed specifically M14 (Fig. 3B).

Table 1. Rank of each genotype for severity of traits controlled by o2 (1 = greatest difference between o2 and wild-type; 8 = least difference between o2 and wild-type).

Trait	A619	B14A	B45	B46	B57	B66	B73	M14
Lysine (g/100g)	3 (tie)	3 (tie)	7 (tie)	1	4	7 (tie)	7 (tie)	8
Kernel density	4	3	5	1	6	2	7	8
Seed weight	8	1	7	2	6	3	4	5
Shoot:root ratio	7	3	4	1	5	6	2	8
Warm germ	7	8	3	1	5	2	5	6

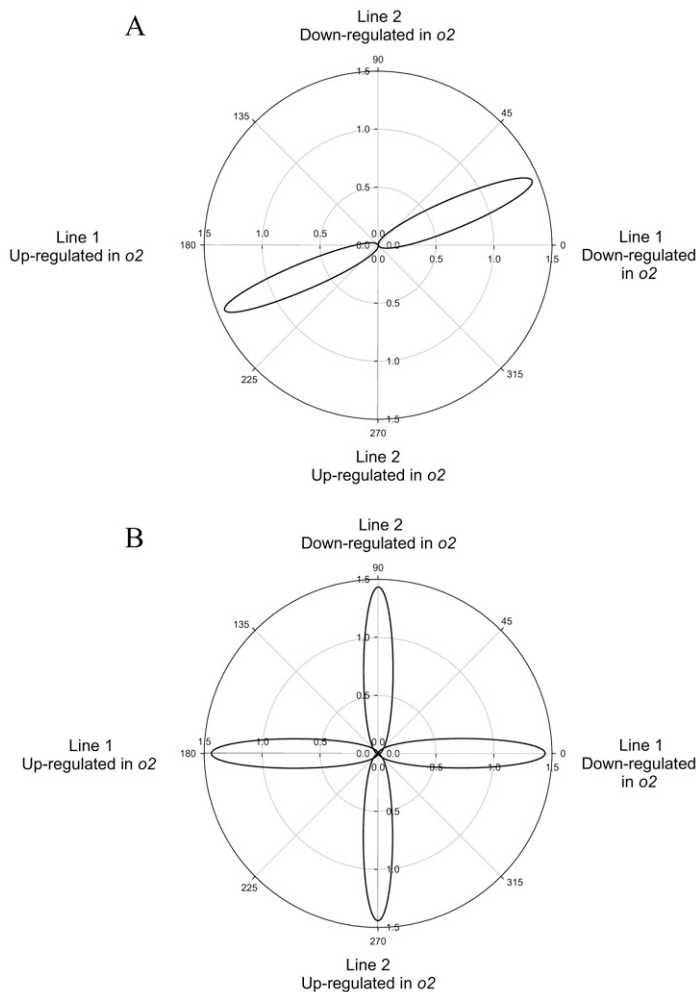


Figure 3. Graphical approach to illustrating degree of differential expression in two genetic backgrounds. The degree of differential expression [$\log(\text{wild-type}/o2)$] of transcripts likely to be differentially expressed (those with low P -values) is plotted as XY data, with each axis representing the degree of differential expression for an $o2$ vs. wild-type comparison in one genetic background. Ellipses indicate the regions where transcripts would be expected to cluster given the following hypotheses to explain differences in phenotypic severity in different genetic backgrounds. A. Hypothesis 1. The degree of differential expression hypothesis suggests one set of transcripts will be uniformly and differentially regulated, more so in one genetic background than the other. This difference in transcript levels causes the difference in phenotypic severity. B. Hypothesis 2. The genotype-specific differential expression hypothesis suggests certain transcripts will be differentially expressed in one genetic background while others will be differentially expressed in the other genetic background. The difference in phenotypic severity is determined by the specific transcripts that are differentially expressed in each genetic background.

Comparative Transcriptional Profiling

To determine the extent to which these two hypotheses was occurring, we compared differential (wild-type vs. $o2$) transcript accumulation in B46/B46 $o2$ and in M14/M14 $o2$ by differential transcript profiling. A maize Unigene cDNA chip contain-

ing 7466 maize cDNA clones spotted on glass slides was used for this purpose.

To determine the degree to which our two hypotheses explain the difference in severity of the $o2$ mutation in the inbred lines B46 and M14, we plotted the degree of differential expression of the transcripts most likely to be differentially expressed (P value $< .001$ in the B14, M14 or pooled genotypes comparisons) in B46 vs. the degree of differential expression of these transcripts in M14 (Fig. 4). Transcripts plotted this way clustered along three rays of the plot. To determine an average radial value for each cluster, a histogram of the radial values of all transcripts on the plot was constructed and Gaussian distributions were fit to each region of the histogram containing a peak (Fig. 4). The radial values of these peaks gave an approximate mean radial value for each cluster. These values were 19, 188, and 247 degrees for the three clusters observed in this analysis. Comparing these radial positions to the expected positions of clusters given each of our hypotheses (Fig. 3) allowed us to determine the extent to which each hypothesis was functioning. The cluster centered on 19 degrees was consistent with the uniform change in degree of differential expression hypothesis, however, because there were few symmetrically oriented transcripts (which would be at 199 degrees), it appeared that this hypothesis fits best to transcripts that were downregulated by $o2$.

In contrast, the cluster centered at 247 degrees, because it is close to 270 degrees, is most consistent with the gene-specific transcript hypothesis. The cluster at 188 degrees could support either hypothesis because this cluster is in a position intermediate between where genotype specific transcripts would be (180 degrees) and where a cluster symmetrically oriented to the 19 degree cluster would be (199 degrees). The lack of clusters near 90 and 0 degrees however, indicated that the genotype specific hypothesis may be most applicable to genes that were upregulated in $o2$.

Examination of the positions of the transcripts identified as differentially expressed in the pooled genotypes experiment revealed that these transcripts clustered along two distinct rays at 33 and 196 degrees (Fig. 4B). This was not a perfect match to the predicted results of 45 and 180 degrees and may be a manifestation of the uniform change in degree of differential expression hypothesis. The radial positions of these clusters indicated

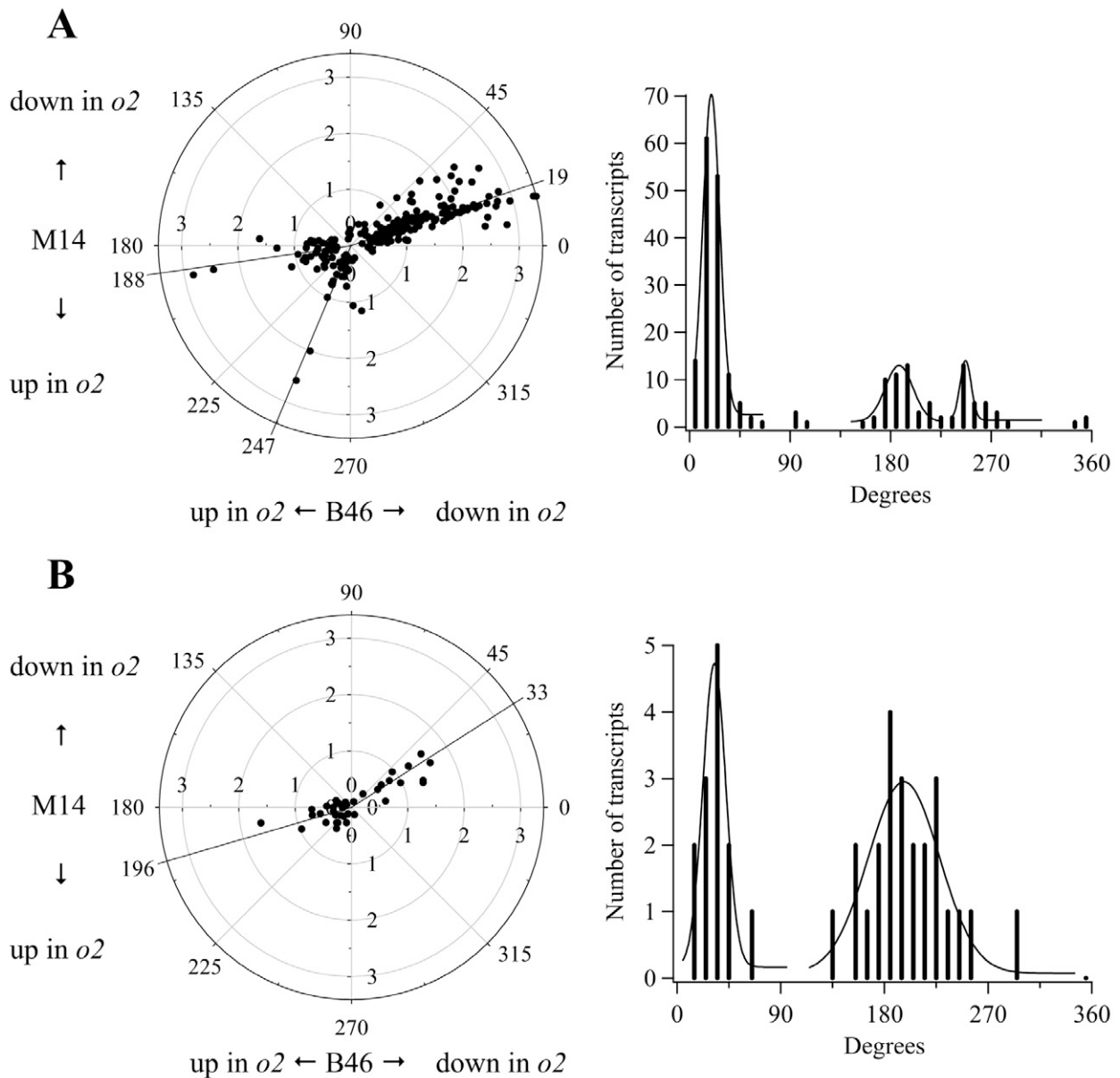


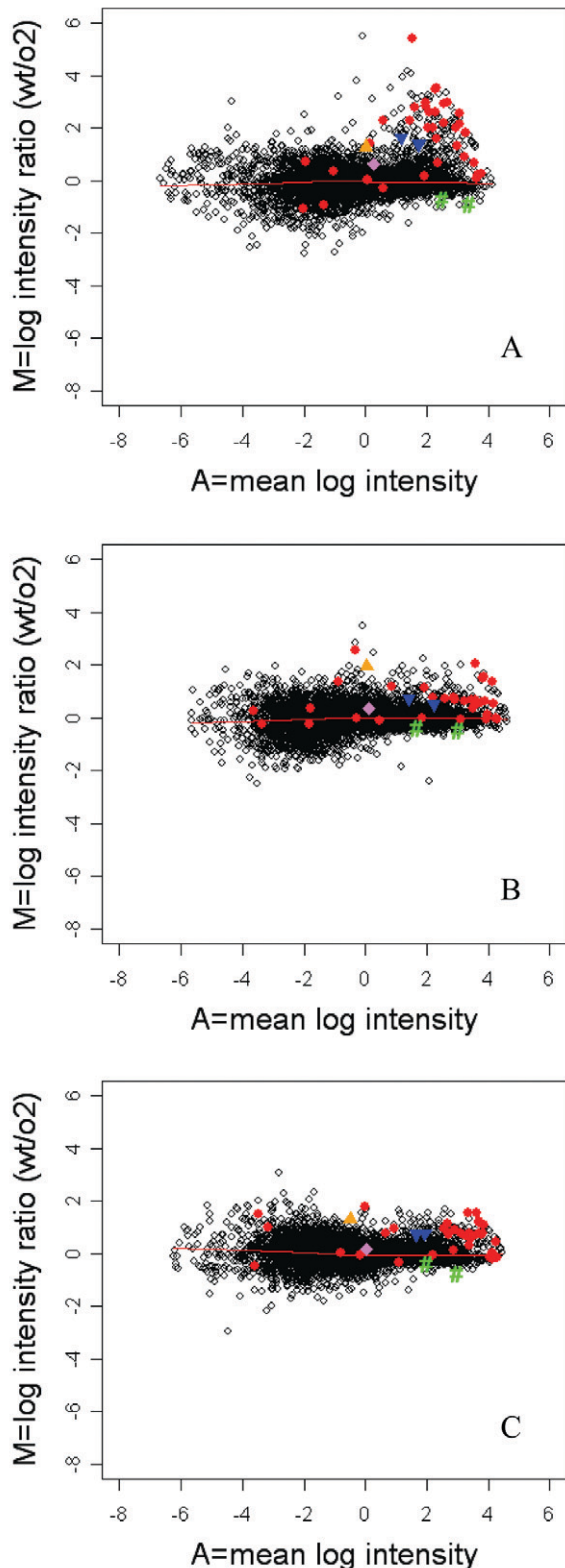
Figure 4. Degree of differential expression [$\log(\text{wild-type}/o2)$] in B46 (x axis) and M14 (y axis). Histograms on the right give the number of transcripts in each bins (defined as a 10 degree segment of the polar plot). Gaussian distributions were fit to each peak in the histogram, and the value of the peak of the fitted distribution is indicated with a line on each polar plot. A. Transcripts plotted are those identified as differentially expressed ($P < 0.001$) in the B46, M14 or pooled genotypes comparisons. B. Transcripts plotted are those identified as differentially expressed ($P < 0.001$) in the pooled genotypes comparison.

that these transcripts tended to be more highly differentially expressed in B46 than in M14.

The radial positions of clusters did not match either hypothesis perfectly. For example, clusters were found at 188 degrees rather than 180, and 247 degrees rather than 270, so some combination of the two hypotheses, or a third yet-to-be-identified hypothesis is required to fully explain the result. What is clear, however, is that up- and downregulated genes have different patterns of transcript accumulation. Upregulated genes are more

likely to have a higher degree of differential expression in one line or the other, fitting with the gene-specific transcript hypothesis. Down-regulated genes are more likely to be differentially expressed in both lines, but to different degrees in each line, fitting with the uniform change in degree of differential expression hypothesis.

To explain the observation that the upregulated genes were more likely to be differentially expressed in a genotype-specific manner than downregulated genes, we examined the gene annotations for the



transcripts in each cluster. The cluster of down-regulated genes along the 19 degree ray contained eight transcripts with homology to α -zeins and one with homology to γ -zeins. In addition to zeins, this

Figure 5. Results of transcript profiling. A. B46. B. M14. C: Eight genotypes pooled. Mean values for each transcript on the array is plotted. The signal intensity on the x axis is presented as a deviation from the mean. The red line is the LOWESS normalization curve. Zein transcripts are represented with red circles. The *Opaque2* transcript is represented with a violet diamond. The *b32* transcript is represented with an orange triangle with its point up. Pyruvate orthophosphate dikinase transcripts are represented with blue triangles with their points down. Cytosolic glyceraldehyde-3-phosphate dehydrogenase transcripts are represented with green #.

cluster contained six transcripts with homology to ribosomal proteins, four transcripts with homology to genes involved in signal transduction and genes involved in carbohydrate metabolism including starch branching enzyme I, an alpha-galactosidase, and an alpha-glucosidase. Transcripts with homology to genes involved in amino acid metabolism were prominent in this group, including an amino acid transporter, arginine methyltransferase, branched chain keto-acid dehydrogenase, L-allo threonine aldolase, glutamate synthase, and 2-isopropylmalate synthase.

The functional annotations of transcripts in the cluster of upregulated transcripts at 247 degrees were remarkable because four out of seven annotated transcripts have a corresponding transcript with an identical annotation in the downregulated, 19 degree cluster. A fifth transcript with a corresponding upregulated transcript in the downregulated cluster occurred in the 188 degree cluster. Thus, there were five examples of transcripts with homology to accessions with identical annotations that were differentially expressed in the opposite direction. These examples included transcripts with homology to genes involved in cytokinin response, transcripts with homology to L-allo-threonine aldolase, transcripts with homology to NADH-dependant glutamate synthases, and transcripts with homology to 2-isopropylmalate synthase. Most notable were transcripts with homology to starch branching enzyme I. One transcript was slightly upregulated and the other was slightly downregulated. This could result in a shift in prominence from one isoform of starch branching enzyme to another, which could contribute to the change in starch structure observed in *o2* mutants (Gibbon et al., 2003). A second explanation for similar transcripts being regulated in different directions may be that the level of one transcript was altered by the *o2* mutation and the level of a transcript with a similar function was altered to compensate for the change.

To put these results in context, it is important to consider some of the trends we observed in the

B46, M14 and pooled genotypes comparisons. In contrast to the pooled genotypes comparison, in both the B46 and M14 comparisons more transcripts were down- than upregulated by *o2*, consistent with its role as a transcriptional activator. B46 had a larger range of intensity ratios (wild-type/*o2*) than M14, indicating that, in general, transcript levels were changed to a greater extent by the *o2* mutation in B46 than in M14 (Fig. 5). Differentially expressed genes tended to be changed in the same direction (up- or downregulated) in the B46 and M14 comparisons.

To compare the prevalence of differentially expressed transcripts in the B46 and M14 comparisons, we computed P-values for gene-specific tests of differential expression (wild-type vs. *o2*) in each background. Regardless of the P-value threshold used to declare differential expression, B46, the genotype with more severe *o2* phenotypes, had more transcripts identified as being differentially expressed than M14, the genotype with less severe *o2* phenotypes. B46 also had more differentially expressed transcripts than the pooled genotypes experiment at all P-values examined, while the number of transcripts identified as differentially expressed in M14 and the pooled genotypes experiment was similar at most P-values (Fig. 6). Furthermore, a method for estimating the proportion of differentially expressed transcripts from an observed P-value distribution was applied to the P-values from each background. Estimates of 25.0 and 14.8% for the proportion of differentially expressed transcripts among all transcripts profiled were obtained for the B46 and M14 backgrounds, respectively. This observation was consistent with the gene-specific transcript hypothesis; although it could be that the M14 transcripts corresponding to the differentially expressed transcripts in B46 were differentially expressed to a lower degree, and therefore were not identified as likely to be differentially expressed. Therefore, this data alone does not allow us to distinguish between our two hypotheses.

Verification of Differential Expression

One way to assess the quality of this data was to examine estimated probabilities of differential expression for genes that have been previously reported to be differentially expressed in *o2* vs. wild-type endosperm (Fig. 7). To estimate the probability that a given gene was differentially expressed, the observed P-value distributions from our three microarray experiments

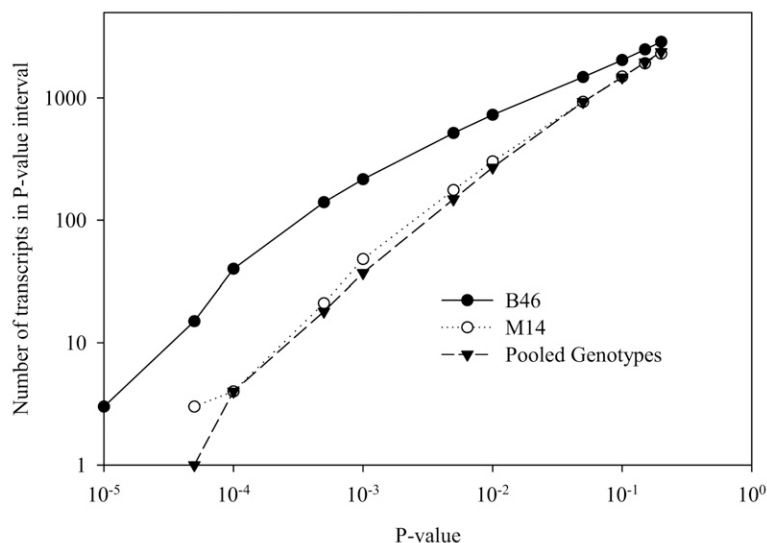


Figure 6. Comparison of the number of transcripts likely to be differentially expressed (wild-type vs. *o2*) for the indicated genotypes at a given probability (P value). The “pooled” line is for the comparison of eight wild-type genotypes pooled vs. a pool of their *o2* counterparts.

were used to estimate the “posterior probability of differential expression” (PPDE) (Allison et al., 2002) for each transcript in each experiment. Pyruvate orthophosphate dikinase (PPDK) (Bass et al., 1992) and *b32* (Muller and Knudsen, 1993) have been reported to be downregulated by *o2*, and the cytosolic glyceraldehyde-3-phosphate dehydrogenase (Maddaloni et al., 1996) and elongation factor 1 α (Carneiro et al., 1999; Habben et al., 1995) are known to be upregulated in *o2* mutants. Of course, the *o2* transcript is expected to be differentially expressed as well. In the B46 comparison, all of these genes probably (> 50%) differentially expressed. Several of these genes were not likely to be differentially expressed in the pooled genotypes or the M14 comparisons. This could be a reflection of differences in expression between the genotypes used in the different studies. The obvious exception to this was the *o2* transcript. The lack of differential expression of this transcript was likely caused by the low levels of this transcript which made determination of differential expression difficult.

A second method to verify the quality of these data was to compare the levels of zeins observed in B46 and M14 (Fig. 2) with the transcript levels of these zeins. The degree of differential expression of the α -zein genes was higher in B46*o2* than in M14*o2*. The expression of twelve α -zein genes in B46*o2* was decreased more than eight-fold relative to the wild-type, whereas expression of eleven α -zein genes was less than three-fold different in M14 compared to the M14*o2*. The difference in zein differential expression between the B46, M14 and pooled genotype comparisons was apparent when comparing the

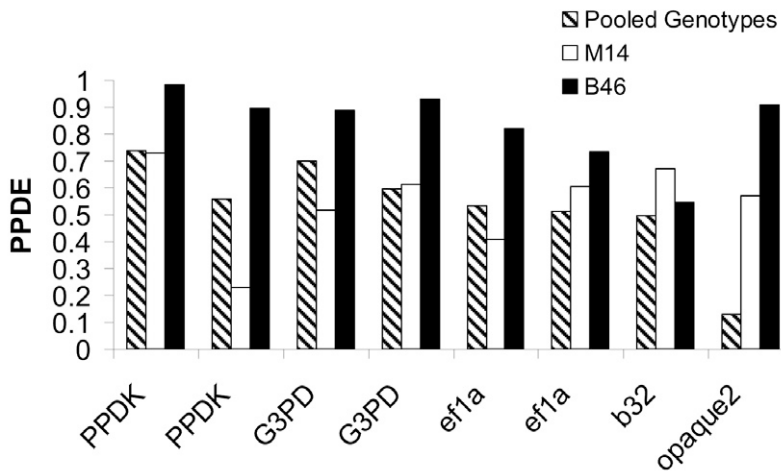


Figure 7. Posterior probabilities of differential expression (PPDE) for specific transcripts estimated from our three transcript profiling experiments. These transcripts have been reported to be differentially expressed in previous investigations. PPDK, orthophosphate dikinase; G3PD, Glyceraldehyde-3-phosphate dehydrogenase; EF1a, Elongation factor 1 alpha; B32, ribosome inactivating protein.

distributions of posterior probabilities of differential expression of the zeins. In B46, all of the zeins were likely to be differentially expressed, while in the M14 and pooled genotypes, some of the zeins were not likely to be differentially expressed (Fig. 8). In general, these observations were consistent with the zein levels in the M14 and B46 backgrounds (Fig. 2).

Finally, to characterize the *o2* allele in the B46 and M14 inbred lines and to confirm the hybridization results, the *o2* transcript and four ESTs were analyzed by reverse transcriptase-PCR analysis (Fig. 9). The 18S rRNA was used as an internal control. No band associated with the *o2* gene was observed for either B46*o2* or M14*o2*. Wild-type B46 had a much stronger *O2* band than did the wild-type M14. Correlations between the probabilities of differential expression and the band intensities were good for B46. In M14, the *o2* transcript was predicted to be not differentially expressed, while it appeared to be differentially expressed in the PCR experiment. The other transcripts were not strongly predicted one way or the other, so it was difficult to conclude if these experiments were in concordance.

Discussion

The *opaque2* mutation is used in breeding programs around the world to produce maize varieties with improved nutrition. The poor agronomic quality of *opaque2* grain is a major obstacle in these programs, and a great deal of effort has been required to overcome this obstacle. This has been accomplished by developing *o2* varieties lacking severe *o2* phenotypes. The severity of the phenotypic effects of the *o2* mutation has been shown to

be background-dependent in numerous reports (Bernard et al., 1994; Ciceri et al., 2000; Schmidt, 1993; Paez et al., 1969; Moro et al., 1996; Wang and Larkins, 2001) and this has been clearly confirmed in this report. These genetic background effects are useful to breeders because they provide a source of phenotypic variation from which to select desirable germplasm, however they complicate efforts to understand the mechanism by which the *o2* gene conditions its characteristic phenotypes. This is because a change observed in one genetic background may occur only in that genetic background and may not be a required component of the *o2* response.

To focus on effects that tend to be present in multiple backgrounds, we pooled mRNA from eight paired wild-type and *o2* inbred lines for microarray analysis. Transcripts that

were differentially expressed in this comparison are likely to be important for understanding the general mechanism of *O2* gene expression regulation because they are likely to be differentially expressed in several genetic backgrounds. Thus, we believe our pooled design has been useful for enriching the pool of wild-type vs. *o2* differences for transcripts that are important in understanding the general mechanism of *o2* function. While the pooling strategy is economical compared to separate analysis of each genotype, pooling does not provide information about the variance of differential expression across genotypes that separate analyses would.

The core set of 37 transcripts contained transcripts with functional annotations related to signal transduction, enzymes and DNA-binding proteins, while it lacked zeins, probably because they exhibited genotype specific expression. The identities of these transcripts suggest that signal transduction may be a central component of the *o2* response in the genetic backgrounds studied here. The transcripts identified in this set would be good candidates to follow up on in studies of *o2* function.

A previous study (Damerval and Le Guilloux, 1998) identified differentially expressed (*o2* vs. wild-type) polypeptides in seven genetic backgrounds. Of 160 polypeptides that were differentially expressed in one genetic background, 36 were differentially expressed in all seven genetic backgrounds. In our analysis, transcripts encoding similar polypeptides to these 36 were probably differentially expressed in most cases (PPDE > 50%), however none of these transcripts were on our list of the 37 transcripts most

likely to be differentially expressed in the pooled transcripts experiment. This discrepancy may be because the polypeptide analysis used is more likely to identify abundant proteins, and because about 7 times as many transcripts were examined than polypeptides we were able to identify differentially expressed transcripts with expression levels too low to be detected by polypeptide analysis.

In an effort to better understand how the specific genetic backgrounds result in expression of *o2* phenotypes with different severities, we characterized wild-type vs. *o2* differential expression of transcripts in B46, a genetic background with severe *opaque2* phenotypes and in M14, a genetic background with minimal *opaque2* phenotypes. Thus, two sets of wild-type vs. *o2* transcript data were generated. These two sets were compared to identify differences in gene expression in the two genetic backgrounds. B46, a genotype with severe *opaque2* phenotypes, had more differentially expressed transcripts than M14, a genotype with minimal *opaque2* phenotypes.

We proposed and tested two hypotheses to explain how transcripts could be differentially expressed such that different genetic backgrounds exhibit different phenotypic severities. One hypothesis is that differential expression in the two backgrounds is gene specific and B46 has differentially expressed transcripts that condition severe phenotypes, while M14 has differentially expressed transcripts that condition mild phenotypes. This hypothesis reflects the possibility that relatively few discrete loci have major effects on the phenotypic severity in *o2* plants, and is supported by the identification of QTL that control modifier loci in a population derived from a cross between a modified *o2* genotype and an unmodified *o2* genotype (Lopes et al., 1995). Transcripts that are close to the axes but far from the origin of Fig. 4 support this hypothesis.

A second hypothesis to explain the severity of the *o2* phenotypes is that phenotypic severity depends on the *degree* of differential expression of a set of transcripts that are differentially expressed in both genetic backgrounds. This hypothesis reflects the possibility that many loci control phenotypic severity in *o2* plants, with no individual loci having major effects. Among the transcripts that were differentially expressed in both B46 and M14, the degree of differential expression tended to be greater in the B46 background. Transcripts located between 0 and 45 degrees and between 180 and 270 degrees in Fig. 4 support this hypothesis.

One difficulty with interpreting these results is that we cannot assign clear boundaries that delineate the differences between our hypotheses. Thus, judgment is required in determining which hypothesis each gene

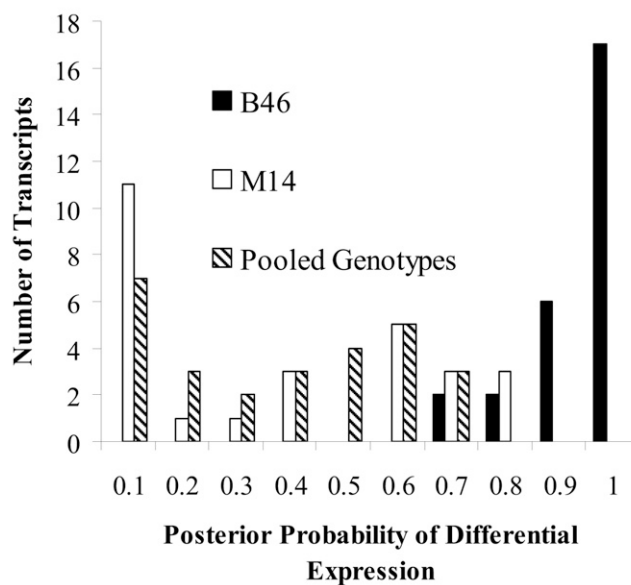


Figure 8. Histogram of estimated posterior probabilities of differential expression for zein transcripts from three transcript profiling experiments.

cluster supports. With this caveat in mind, an intriguing possibility is that the uniform change in differential expression hypothesis fits better with transcripts that are downregulated by *o2*, while the genotype-specific transcript hypothesis fits better with genes that are upregulated by *o2*. Moreover, five of the transcripts that are upregulated by *o2* have homologs in the downregulated set. This suggests that transcript levels may be controlled to compensate for changes in levels of other transcripts. These upregulated transcripts tended to be more highly differentially expressed in M14, and may explain in part the minimal severity of the *o2* phenotypes in this line. These observations lead to a novel hypothesis in need of further investigation, namely that the set of transcripts directly affected by *o2* are predominantly downregulated and consistent across genetic backgrounds. The downregulation of these transcripts causes a compensatory response such that transcripts indirectly affected by *o2* are predominantly upregulated and are specific to the genetic background. These upregulated transcripts, in turn, determine the severity of the phenotypic response to *o2*.

Expression of the *O2* gene in M14 wild-type was lower than in B46 wild-type (Fig. 9). The *o2* transcript was not detectable in either genetic background. Thus, the reduction in *O2* transcript level on conversion to *o2* was greater in B46 than in M14, correlating with the phenotypic severities in these two genetic backgrounds. It may be that B46 requires higher levels of *O2* to maintain a wild-type phenotype than does M14, therefore the loss of the *O2* transcript has a greater impact reflected in a more severe phenotype.

Transcript	B46		M14	
	PCR result	PPDE	PCR result	PPDE
18S rRNA				
<i>Opaque2</i>		0.91		0.07
<i>Wali7</i>		0.95		0.50
AW076489		0.98		0.62
AI745822		0.96		0.53
AI677412		0.97		0.84

Figure 9. Ethidium bromide stained agarose gels showing RT-PCR confirmation of microarray results and the estimated posterior probability of differential expression (PPDE) for that pair of transcripts. Negative images are presented so darker bands contain more DNA. In each PCR result, the band on the right is from *o2* RNA and the band on the left is from wild-type RNA.

In summary, a core set of transcripts was identified by averaging differential expression across genotypes. These transcripts were differentially expressed in two genetic backgrounds that differed in severity of the *o2* mutation. In these two genetic backgrounds, downregulated transcripts tended to be differentially expressed to a different degree in each genetic background, with the difference in degree of differential expression being fairly uniform across transcripts. In contrast, upregulated transcripts tended to be more strongly differentially expressed in one genotype or the other and sometimes duplicated a functional annotation found in the downregulated transcripts. Thus, genotype-specific differences in the *o2* mutation included a combination of mechanisms involving both uniform and gene-specific differences in transcript levels that may result in compensation for changes caused by the mutation.

Materials and Methods

Plant Material

Eight maize inbred lines, A619, B14A, B45, B46, B66, B57, B73, and M14, and their *opaque2* conversions were developed by Dr. Peter Loesch (Iowa State University), maintained at Iowa State University, and grown at the Iowa State University Agronomy Farm (Boone, Iowa) during the summer of 2002. The paired inbred lines were grown in adjacent rows and self-pollinated by hand.

Analysis of Grain Traits

Tissue (100 mg) of ground mature kernels from each of the sixteen lines of wild-type and *opaque2* mutants was analyzed using the AOAC standard method for complete amino acid analysis (AOAC International, 2002). Five mature kernels were collected from each of the wild-type and *opaque2* mutant lines and their density was determined in duplicate using a gas pycnometer and 3.5 cm³ container (Model AccuPyc 1330, Micromeritics, Norcross, GA) (Chang, 1988; AOAC International, 2002). Viability and vigor were measured by conducting standard warm and cold germination tests (AOSA, 2003), respectively, on three lots of 10 seeds each from each of the wild-type and mutant lines. In addition, the dry weights of normal shoot and root tissue taken following cold germination were determined and are reported as a ratio.

Analysis of Zeins

Mature endosperm fractions were extracted with 70% (v/v) ethanol, 5% (v/v) 2-mercaptoethanol, and 0.5% sodium acetate in 1.5 mL polypropylene tubes by shaking 2 h at room temperature. Alcohol-extracted proteins were diluted four-fold with extraction buffer and separated by HPLC using a RP-C18 column. Elution was performed using a gradient of solvents A (99.9% (v/v) acetonitrile plus 0.1% (v/v) trifluoroacetic acid) and B (99.9% water plus 0.1% (v/v) trifluoroacetic acid) (Wilson, 1991; Paulis et al., 1991). Samples (100 μ L) were injected into a BioCad HPLC system (Applied Biosystems, Foster City, CA). The UV absorbance of the column effluent was monitored at 210 nm.

Isolation of mRNA and preparation of labeled targets

For microarray expression profiling, endosperm tissue was dissected from field-grown ears at 14 DAP. Plants were randomly selected and endosperm tissue was harvested at midday, frozen in liquid nitrogen, and stored at -80°C . mRNA was extracted from the frozen immature endosperm tissue with the PolyATract System 1000 mRNA isolation kit (Promega, Madison, WI) according to the manufacturer's instructions. The mRNA was directly labeled with Cy3- or Cy5-dUTP (Amersham Pharmacia Biotech, Piscataway, NJ) using reverse transcription. The reaction mixture (25 μ L) was composed of 4 μ g mRNA, 2 μ L dNTP mix (10 mM each of dA, dC, dG, and 2 mM dT), 2 μ L Cy3- or Cy5-dUTP, and 2 μ L primer oligo dT₂₃ (0.5 μ g/ μ L). The mixture was incu-

bated at 65°C for 10 min and then transferred to ice. Finally, 8 μL 5X reverse transcriptase (RT) buffer, 4 μL 0.1 M dithiothreitol (DTT), 2 μL SuperScript II (Invitrogen, Carlsbad, CA), and 1 μL RNase inhibitor (1 U/ μL) were added. This mixture was incubated at 42°C for 90 min. The polymerase and mRNA were inactivated by adding 5 μL 0.5 M EDTA and 10 μL 1.0 M NaOH and incubating at 65°C for 10 min. The reaction was neutralized by adding 10 μL 1.0 M HCl. The unincorporated fluorescent nucleotides were removed by ultra-filtration (Microcon-YM3, Millipore, Billerica, MA). The filters were washed four times with 400 μL TE buffer (10 mM Tris-Cl, pH 7.5, 1 mM EDTA) and centrifuged at 12 000 $\times g$ for 20 min. The purified cDNA was eluted with 20 μL TE by inverting the spin column and centrifuging at 12 000 $\times g$ for 5 min. The quality of labeled first strand cDNA was evaluated by running 1 μL of the purified cDNA on a 1% agarose gel which was scanned using a Typhoon fluorescence scanner (Amersham Pharmacia Biotech, Piscataway, NJ).

Microarray Hybridization

A triplicate dye-swap design was used for the microarray experiment. For each single dye-swap experiment two slides were used. Three pairs of wild-type and *o2* mutant ears were randomly selected. Within each pair, the mRNA of pooled *opaque2* mutant endosperms from one ear was labeled with Cy3-dUTP, whereas the mRNA of pooled wild-type endosperms from the other ear was labeled with Cy5-dUTP. The two labeled targets were mixed together and hybridized to one slide. A second labeling of each batch of mRNA was performed with the dyes swapped relative to the first labeling. mRNAs from the three pairs of ears were labeled separately to give a total of six hybridizations for each comparison.

Slides of the maize Unigene microarray were obtained from the microarray laboratory of the Maize Gene Discovery Project (Fernandes et al., 2002). The microarray consisted of 7466 maize cDNAs spotted in duplicate on a glass slide along with controls consisting of human and *Arabidopsis* cDNA clones that do not hybridize to maize sequences. Labeled cDNA was hybridized to these slides according to the protocol recommended by the Maize Gene Discovery Project (www.maizegdb.org/documentation/mgdp/microarray/protocols.php; verified 18 Aug. 2006). Briefly, before hybridization, slides were rehydrated by placing them face down over a 42°C water bath for 5 to 10 s, then drying briefly on a 65°C heating block for 3 to 10 s until the moisture was no longer visible. The DNA was UV-cross-linked at 65 mJ of 254 nm UV light with a Stratlinker (Stratagene, La Jolla, CA). The slides were immersed in 1% sodium dodecyl sulfate (SDS) for 5 min to remove unbound nucleic acids

and in 95°C water for 2 min to denature the DNA, and then dried with a stream of nitrogen. For each slide, 50 μL of hybridization solution were prepared by mixing 3 μL Liquid Block (Amersham Pharmacia Biotech, Piscataway, NJ), 5 μL 20 \times saline-sodium citrate buffer (SSC), 2 μL 2% SDS, 20 μL Cy3-labeled probe or 20 μL Cy5-labeled probe. The mixture was denatured at 95°C for 2 min, transferred to ice, then centrifuged briefly in a microcentrifuge. The mixture then was placed on the slide and covered with a cover slip (Hybrislips Z36, Sigma-Aldrich, St. Louis, MO). The slide was placed in a microarray hybridization chamber (ArrayIt Hybridization Cassette, TeleChem International, Sunnyvale, CA). Hybridization was performed in a water bath at 65°C for 8 to 9 h. After the hybridization, the slide was washed in 2 \times SSC and 0.5% SDS at 55°C for 5 min, in 0.5 \times SSC at room temperature for 5 min, followed by 0.05 \times SSC at room temperature for 5 min and then dried with a stream of nitrogen as before.

The slide was scanned using a laser scanner (Scan-Array 5000, GSI Lumonics- Packard BioScience, Billerica, MA) for both channel 1 (Cy3) and 2 (Cy5) at 10 μm resolution. The channel 1 and channel 2 images were analyzed using ImaGene 4.1 software (BioDiscovery, Marina Del Rey, CA) to obtain average signal and background intensities for each spot.

Microarray Data Analysis

The raw data from three sets of comparisons, B46/B46*o2*, M14/M14*o2*, and pooled genotypes wild-type/pooled genotypes *o2*, consisting of average signal and background for each spot from a total of 18 slides (6 slides for each comparison) were integrated into one data file. Before further analysis, the empty and control spots were removed. The background of each spot was subtracted from the signal and the background-corrected signal intensities of duplicate spots within each slide were averaged. This value was set to zero if negative. One was then added to each averaged, background-corrected signal and these data were transformed using the natural log function. LOWESS normalization was used to remove intensity-dependent dye bias (Yang et al., 2002). After LOWESS normalization, the treatment difference (log wild-type/*o2*) of each gene for each rep (dye-swap) within each comparison was averaged to produce a total of nine independent differences (three for each background). For a given transcript, let Y_{ij} denote the j^{th} difference in the i^{th} background. The following linear model was fit separately for each transcript.

$$Y_{ij} = \mu_i + e_{ij} \quad i = 1, 2, 3 \text{ and } j = 1, 2, 3$$

where μ_1 , μ_2 , and μ_3 denote the gene-specific mean differences in the three backgrounds and e_{ij} denotes

independent, normally distributed residuals with mean 0 and a variance σ^2 specific to the transcript. A P-value for the test of $H_{0i}: \mu_i = 0$ was computed for each background and transcript based on the t-statistic with 6 degrees of freedom.

Next, a mixture of a uniform and a beta distribution was fit to the observed P-value distribution for each background as described by Allison et al. (2002). The fitted mixture distribution was used to estimate the posterior probability of differential expression for each gene in each background and the proportion of transcripts differentially expressed in each background using the method of Allison et al. (2002).

RT-PCR

DNase-treated polyA mRNA (0.5 μ g) from 14 DAP endosperm of wild-type and mutant kernels were used for first strand cDNA synthesis. Each reaction (40 μ L) contained 2 μ L dNTP mix (10 mM each dA, dC, dG, dT), 2 μ L primer oligo dT₂₃ (0.5 μ g/ μ L) (Invitrogen, Carlsbad, CA), 8 μ L 5X RT buffer, 4 μ L 0.1 M DTT, 2 μ L SuperScript II (Invitrogen, Carlsbad CA), and 1 μ L RNase inhibitor (1 U/ μ L). The reaction mixture was incubated at 42°C for 90 min. PCR reactions were performed with gene specific primers for: 18s *rRNA* (forward primer AGTTTGAGGCAATAACAGGTCT, reverse primer GATGAAATTTCCCAAGATTACC); *Opaque2* (forward primer AAGATCAACGACGCTAACGTC, reverse primer GACGACGGCACTGATGAG); *Wali7* (forward primer TATCATCTCCTCCCACCCG, reverse primer ACGCAACACACAACACATCC); AI745822 (forward primer GAAGAGA-ACTCCCAATACGGC, reverse primer CGATTCTGCTACCAAACG); AI677412 (forward primer TTCTGCTGTGGTTGTTTCGC, reverse primer AGGAAAGGGAAAGGGTGC); and AW076489 (forward primer CTACCCAC-TACCCAGCATTTG, reverse primer ACGAAC-GGGATTAGCGG). PCR was performed using JumpStart RedTaq Ready Mix (Sigma-Aldrich, St. Louis, MO) according to the manufacturer's directions (*Wali7*: 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min with 25 cycles; all others: 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min with 30 cycles) in a MJ Research PTC-200 (Watertown, MA) thermal cycler. Amplified products were separated on 4% Metaphor agarose gels (BioWhittaker Molecular Applications, Inc, Rockland, ME) in TBE buffer.

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