Pyrophosphate-Dependent Fructose-6-Phosphate 1-Phosphotransferase Induction and Attenuation of Hsp Gene Expression during Endosperm Modification in Quality Protein Maize

Xiaomei Guo, Kyla Ronhovde, Lingling Yuan, Bo Yao, Madhavan P. Soundararajan, Thomas Elthon, Chi Zhang, and David R. Holding*

Department of Agronomy and Horticulture (X.G., K.R., L.Y., T.E., D.R.H.) and School of Biological Sciences (B.Y., C.Z.), Center for Plant Science Innovation, and Department of Biochemistry (M.P.S.), University of Nebraska, Lincoln, Nebraska 68588

Quality Protein Maize (QPM) is a hard-endosperm version of the high-lysine opaque2 (o2) maize (Zea mays) mutant, but the genes involved in modification of the soft o2 endosperm are largely unknown. Pyrophosphate-dependent fructose-6-phosphate 1-phosphotransferase (PFP) catalyzes the ATP-independent conversion of fructose-6-phosphate to fructose-1,6-bisphosphate in glycolysis. We found a large increase in transcript and protein levels of the α-regulatory subunit of PFP (PFPα) in QPM endosperm. In vitro enzyme assays showed a significant increase in forward PFP activity in developing endosperm extracts of QPM relative to the wild type and o2. An expressed retrogene version of PFPα of unknown function that was not up-regulated in QPM was also identified. The elevated expression levels of a number of ATP-requiring heat shock proteins (Hsps) in o2 endosperm are ameliorated in QPM. PFPα is also coinduced with Hsps in maize roots in response to heat, cold, and the unfolded protein response stresses. We propose that reduced ATP availability resulting from the generalized Hsp response in endosperm are ameliorated in QPM. PFP in QPM was also identified. The elevated expression levels of a number of ATP-requiring heat shock proteins (Hsps) in genes involved in modification of the soft o2 endosperm and in South Africa (Geever and Lake, 1992) led to the development of hard-kernel o2 varieties that retained the high levels of Lys and Trp (Paz et al., 1969; Ortega and Bates, 1983). These varieties, known as Quality Protein Maize (QPM), have been introgressed into many high-yielding maize inbreds and hybrids adapted to diverse growing conditions and are contributing to reducing protein deficiency disorders in humans and to more efficient livestock feeding. Despite the potential of QPM, it remains an unutilized resource in the United States. o2 modifier genes (QPM genes) and the mechanisms by which they convert soft o2 to a vitreous phenotype are unknown, and mapping efforts have indicated that they are multiple in number (Holding et al., 2008, 2011). The complicated genetic basis of QPM, as well as the need to continuously select for the homozygous mutant o2 allele, have impeded breeding efforts and the incorporation of QPM into agriculture in the United States and worldwide.

QPM has low levels of α-zeins, like o2, but several studies have shown that the 27-kD γ-zein is increased 2- to 3-fold (Wallace et al., 1990; Geetha et al., 1991; Lopes and Larkins, 1991; Holding et al., 2011), and the degree of endosperm vitreousness closely correlates with the level of 27-kD γ-zein protein (Lopes and Larkins, 1991). Furthermore, the 27-kD γ-zein gene maps to a major chromosome 7 quantitative trait locus (QTL) for endosperm hardness in QPM (Holding et al., 2008, 2011), and it was recently shown that γ-zein is essential for endosperm modification in QPM (Wu et al., 2011).
et al., 2010). Since the 27-kD γ-zein is thought to initiate protein body formation, it has been suggested that this increase causes the observed elevation in protein body number in QPM (Lopes and Larkins, 1995; Moro et al., 1995), which in turn increases protein cross-linking and restores kernel hardness.

Mapping and transcriptional profiling have identified at least three QPM QTLs on chromosomes 1, 7, and 9 and a number of candidate genes for these QTLs (Holding et al., 2008, 2011). A gene encoding the regulatory α-subunit of pyrophosphate-dependent fructose-6-phosphate 1-phosphotransferase (PFPα), which maps close to the chromosome 9 QTL, was identified in a microarray experiment as having a 5-fold increased expression in endosperm of modified versus nonmodified o2 (Holding et al., 2008). Real-time quantitative reverse transcriptase (qRT)-PCR showed a 16-fold increase in PFPα in QPM over the wild type and, furthermore, that expression in o2 endosperm was substantially reduced compared with the wild type (Holding et al., 2011). Recombinant inbred lines (RILs) resulting from a cross between W64Aa2 and K0326Y, a QPM inbred developed in South Africa (Geevers and Lake, 1992), showed that high PFPα expression often coincided with endosperm modification, whereas nonmodified, opaque endosperm RILs exhibited low-level expression like the o2 parent (Holding et al., 2011). However, since we showed that at least partial modification is possible in the presence of low, o2-like PFPα expression (Holding et al., 2011), its high-level expression may not be absolutely required in QPM. In this paper, we describe work to characterize the physiological significance of the increase in PFPα, which may lead to increased glycolytic flux, which may in turn play a role in amelioration of the o2 phenotype in QPM endosperm.

The PFP enzyme complex serves as an alternative, non-ATP-requiring enzyme to ATP-dependent phosphofructokinase for catalyzing a major rate-limiting step in glycolysis (Fru-6-P to Fru-1,6-bisP; Fig. 1) and is located exclusively in the cytosol. In plants, PFP exists as a heterotetramer consisting of two catalytic β-subunits and two regulatory α-subunits, but a variety of other configurations have been reported (Wong et al., 1990; Botha and Botha, 1991). The α-subunit only accumulates during stressful conditions such as phosphate starvation (Theodorou et al., 1992; Huang et al., 2008). Evidence suggests that pyrophosphate (PPI) can serve as an alternative phosphate donor to ATP in plant cells (Jelitto et al., 1992; Davies et al., 1993; Plaxton and Podesta, 2006). Work with anoxia-tolerant rice (Oryza sativa) lines suggested that cellular survival under ATP-deficient, stressed conditions is linked to a switch in energy currency from ATP to PPI manifest by increased activity of PPI-dependent glycolytic enzymes, including PFP and pyruvate orthophosphate dikinase (PPDK; Fig. 1; Huang et al., 2008). Interestingly, the inner maize endosperm environment was shown to be hypoxic and ATP limiting (Rolletschek et al., 2005) and would suggest that glycolysis becomes increasingly important for ATP production as the tricarboxylic acid (TCA) cycle and oxidative phosphorylation slow down. This may in turn suggest that PPI-dependent glycolysis is important during normal endosperm development. PPDK is positively regulated by the O2 transcription factor (Fig. 1; Damerval and Le Guilloux, 1998) and is thus down-regulated in o2 mutants. This could have a negative effect on endosperm glycolysis in o2 endosperm. Testament to the importance of PPDK during late-stage kernel filling is the massive increase in PPDK abundance (Méchin et al., 2007). This raises the possibility that, in addition to defective zein protein body formation, the opaque phenotype results in part from an energy deficiency within the developing endosperm. If normal endosperm development relies on PPI-dependent metabolism, disruption of PPI-dependent enzymes could interfere with the timely and regular progression of endosperm programmed cell death, which in turn could contribute to the opaque phenotype.

Many enzymes that use PPI, including PFP, PPDK, Suc synthase, and UDP-Glc pyrophosphorylase, are induced or up-regulated in response to inorganic phosphate (Pi) deficiency and other ATP-depleting conditions (Plaxton and Podesta, 2006). Most evidence suggests that PFP functions as an adaptive enzyme that enables plants to survive Pi stress by conserving and supplementing ATP. 

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**Figure 1.** Summary of the glycolytic pathway. Reversible enzymes that involve PPI are shown in gray boxes. The solid gray arrow represents transcriptional activation of PPDK by the O2 transcription factor. AGPase, ADP-Glc pyrophosphorylase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PEP, PEP carboxylase; PK, ADP-dependent phosphofructokinase; PPDK, pyruvate kinase.
reserves (Plaxton and Podesta, 2006). ATP, ADP, and other nucleoside phosphate levels quickly fall in response to limiting cytosolic Pi, whereas PPI is largely unaffected (Plaxton and Podesta, 2006). Here, we describe evidence to suggest that cellular stresses in the o2 endosperm that could create an energy crisis may be alleviated in QPM through ATP-independent PFP activity.

RESULTS
cDNA and Genomic DNA Sequencing Identifies an Expressed Retrogene Version of PFPα

Microarray and qRT-PCR experiments comparing endosperm pools of modified (vitreous) and nonmodified (opaque) lines derived from a cross between the South African QPM, K0326Y, and W64Ao2, as well as the parental lines themselves, showed substantial up-regulation of a PFPα gene on chromosome 9.03 in lines exhibiting the modified QPM phenotype (Holding et al., 2008, 2011). In order to verify this differential expression using qRT-PCR, it was necessary to sequence K0326Y and W64A alleles for PFPα so that reliable primers could be designed in regions of 100% conservation. In sequencing full-length cDNAs for PFPα in K0326Y and W64Ao2, we identified two slightly different-length transcripts with very high sequence conservation. The predominant K0326Y transcript contains a 1,869-bp open reading frame (ORF), whereas the other transcript, which we at first believed to be W64Ao2 specific, is 1,764 bp. These genes have a number of polymorphisms, but moreover, the predominant K0326Y transcript has a region of 105 bp encoding 35 amino acids that is not present in the shorter transcript. The predicted proteins from these two transcripts are shown in Figure 2. RT-PCR analysis using primers flanking this 105-bp region showed that both longer and shorter transcripts are present in all genotypes, W64A wild type and o2 as well as K0326Y QPM (Fig. 3A).

To determine if the two PFPα transcripts are the products of differential splicing of a single gene or if they are transcribed from separate genes, we determined the genomic sequence that gives rise to each transcript. Using primers at the extremities of the cDNA sequence, we were able to amplify from genomic DNA a fragment of exactly the same size and sequence as the shorter PFPα cDNA that lacked the 105-bp region and, strikingly, lacked any introns. These primers did not amplify a transcript that could represent the larger PFPα transcript. However, using primers that amplify shorter regions of the gene, we were able to annotate the longer PFPα gene containing the 105-bp region as well as multiple introns that correspond to the GenBank accession (EU957109) and maize genome sequence (GRMZM2G314094; Fig. 2A). In the longer gene, the first two introns are 2,390 and 25,917 bp, respectively, which explains why we were not able to amplify the whole genomic region. The very large second intron size is inferred from the B73 genome and was not sequenced in its entirety for W64A or K0326Y. A very high level of sequence conservation between the longer and shorter transcripts, but their distinct genomic organization with respect to the presence or absence of introns, indicates that the shorter transcript is produced from a gene that is itself a retrotranscribed version of the longer transcript that underwent a reinsertion event into the genome. Furthermore, this gene lost exon 14, since the entire region between the end of exon 13 and the beginning of exon 15, including introns 13 and 14, is absent in the cDNA (Fig. 2B). Thus, the short transcript results from a retrogene that is expressed (Fig. 3, A and B), and its cDNA sequence predicts it to encode a protein that has a 35-amino acid internal deletion relative to the protein from the longer gene but an ORF sequence that shares the same N and C termini as the full-length PFPα protein (Fig. 2C).

Increased PFPα Expression But Not PFPα Retrogene Expression Coincides with Endosperm Modification in QPM

RT-PCR shows that the full-length PFPα is specifically and substantially increased in QPM (Fig. 3A). We also investigated PFPα expression in CM105 QPM, which was generated from the Mexican Pool 33 QPM (Gibbon et al., 2003). After sequencing this cDNA from CM105 wild type, o2, and QPM, we observed the same RT-PCR result, indicating that the increase in the full-length PFPα transcript (Fig. 3B), as well as the existence of the retrogene, are common in QPM backgrounds of completely different pedigree. Using PCR primers entirely within the 105-bp region specific to the full-length transcript as well as primers downstream of the 105-bp region common to both transcripts, we observed a very similar increase in PFPα expression relative to the wild type and o2 using qRT-PCR (Supplemental Fig. S1). This confirmed that the PFPα increase in QPM is a result of specifically increased accumulation of the full-length transcript and not the retrogene.

QTL mapping experiments have identified at least three QTLs associated with endosperm modification in QPM, with locations on chromosomes 1.05, 7.02, and 9.03 (Holding et al., 2008). QTL mapping using K0326Y RILs confirmed that the chromosome 7 QTL is by far the most significant, followed by the chromosome 9 QTL, with the chromosome 1 QTL being slightly below the significance cutoff value for kernel hardness and density (Holding et al., 2011). We obtained developing endosperm for representative RILs that specifically had the K0326Y genotype for the markers flanking each one of these three QTLs (Fig. 3E). The effect of each of the isolated QTLs is manifest by a variable degree of endosperm modification (mature kernel vitreousness; Fig. 3E). In three representative RILs for each QTL, we showed that high-level PFPα expression correlates with both the chromosome 9 QTL and a partially modified, semivitreous phenotype (Fig. 3, C–E). RILs with the W64Ao2 genotype at these QTLs displayed lower than wild-type levels of PFPα expression, similar to the W64Ao2 parent (Fig. 3, C–E).
Increase in the Regulatory PFPα But Not Catalytic PFPβ Subunit Protein Is Specific to QPM Endosperm

To confirm the PFPα differential expression, we made peptide antibodies, including one to detect both the 67.7-kD PFPα and the predicted 63.7-kD PFPα retro-gene protein. However, a protein at the predicted size of PFPα retro-gene was not detected. We also designed a peptide antibody entirely within the 35-amino acid region (encoded by the 105-bp exon 14), which is specific to PFPα but not the retro-gene protein. However, this did not result in a useful antibody. PFPα protein is specifically increased in QPM endosperm compared with the wild type and o2 by mid-endosperm filling (18 d after pollination [DAP]; Fig. 4A). However, during early kernel filling (12 DAP), before the peak in zein accumulation and before the phenotypes of o2 and QPM develop, there was little or no difference in PFPα accumulation between the wild type, o2, and QPM (Fig. 4A). This confirmed earlier RT-PCR data that showed that differential PFPα gene expression is not observed at 12 DAP (data not shown). Although PFPα is abundant in embryo extracts, its accumulation is uniform between the wild type, o2, and QPM (Fig. 4C). High-level endosperm PFPα accumulation shows a good correlation with the vitreous phenotype in RILs derived from the W64A o2 × K0326Y QPM cross (Fig. 4D). Both vitreous RILs shown in Figure 4D are heterozygous for the K0326Y QPM chromosome 9 QTL coordinates, and both opaque RILs are homozygous for the W64A o2 chromosome 9 QTL coordinates. However, both selected semivitreous RILs are homozygous for the W64A o2 chromosome 9 QTL region between markers bnlg127 and umc1771 (Holding et al., 2011) and do not apparently have the K0326Y QPM region between these markers. Thus, it appears that the K0326Y QTL on chromosome 9 may not be necessary for increased PFPα accumulation. Although PFPα may play a role in endosperm modification, along with other factors such as increased γ-zein, the cis- or trans-effect that increases its expression may reside either very close to or outside the coordinates of the chromosome 9 QTL.

Our PFPα antibody frequently recognized a less intense protein band at approximately 50 kD (Fig. 4, C).
and D). This band likely results from another PFPα family member located on chromosome 5. The gene (mRNA accession no. PCO650540), whose transcript is not differentially expressed between the wild type, o2, and QPM (data not shown), encodes a 53-kD protein with 82% amino acid identity with PFPα. The 14-amino acid peptide sequence has one mismatch with that of PFPα. In longer exposure western blots, a larger band of variable intensity is often detected at 100 to 130 kD on the western blots (Fig. 4D). There are no PFP family members in the genome larger than 68 kD. Also, using BLASTn and BLASTp searches of the maize genome using the 42-bp/14-amino acid region, we did not identify any predicted proteins of this larger size. We are investigating the possibility that the larger band could represent a highly stable dimer between two α-subunits or one α-subunit and one β-subunit.

We also made a peptide antibody to the 61.1-kD catalytic PFPβ subunit protein encoded by the GRMZM2G059151 chromosome 6 gene. Western blots with this antibody showed that PFPβ abundance is uniform between genotypes at these early- and mid-endosperm developmental stages (Fig. 4B). The peptide used to make the PFPβ antibody exists in a predicted 47-kD protein encoded by a related mRNA (AY104192), and this likely explains the faint, lower molecular mass band in Figure 4B.

PFP Complex Formation Accompanies High-Level PFPα Accumulation in QPM Endosperm

In the activated form, PFP is reported to exist as a heterotetramer consisting of two catalytic β-subunits and two regulatory α-subunits, and evidence suggests that the α-subunit only accumulates during stressful conditions such as phosphate starvation (Theodorou et al., 1992). A number of different subunit compositions have been detected, including a PFPβ homodimer (Wong et al., 1990; Botha and Botha, 1991). Preliminary western blots of native endosperm extracts of the wild type, o2, and QPM separated on nondenaturing PAGE gels, and probed with the PFPα antibody, showed the presence of a diffuse band at the predicted size of a tetramer (approximately 260 kD) in QPM endosperm that is absent in both wild-type and o2 endosperm (data not shown). To more precisely define the complex formation in QPM endosperm, we separated these native extracts using size-exclusion HPLC followed by SDS-PAGE of the timed elution fractions (Fig. 5). The columns were calibrated using Bio-Rad protein standards, which each eluted over a period of 4 to 5 min. By

Figure 3. Expression of PFPα in maize endosperm. A, RT-PCR of PFPα in W64A wild-type (WT), W64A o2, and K0326Y QPM developing endosperm RNA. Three biological replicate ears are shown for each genotype. Primers flank the 105-bp region absent in the retrogene transcript and are common to both PFPα (top band) and PFPα retrogene (bottom band). B, As for A but comparing CM105 wild type, CM105 o2, and CM105 QPM. C, qRT-PCR of PFPα comparing expression in W64A wild type, W64A o2, K0326Y QPM, and K0326Y by W64A o2 RILs with defined genotypes at chromosome 1, 7, and 9 QPM QTLs (Holding et al., 2011), as shown in D. The PCR product does not span the 105-bp polymorphism, and primer-binding sites are common between PFPα and PFPα retrogene. Differential expression is derived from full-length PFPα (Supplemental Fig. S1). The y axis shows relative expression (log, to the wild type using RBR1 as an internal control gene; Sabelli et al., 2005). Values are derived from three biological replicate ears, and so values represent means of three technical repeats, as described previously (Holding et al., 2011). D, RT-PCR of same lines as in C with genotype at the three QTLs depicted (Holding et al., 2011). A refers to the K0326Y QPM allele, B refers to the W64Ao2 allele, and lines were selected for homozygosity at each of the chromosome 1, 7, and 9 QTLs. Thus, AA BB BB has the homozygous K0326Y QPM genotype only at the chromosome 1 QTL, BB AA BB has the homozygous K0326Y QPM genotype only at the chromosome 7 QTL, and BB BB AA has the homozygous K0326Y QPM genotype only at the chromosome 9 QTL. The chromosome (chr) number for each QTL genotype is shown below the genotype designation. E, Kernel vitreousness/opacity of the RILs shown in C and D using a light box. [See online article for color version of this figure.]
comparing fractions containing PFPα or PFPβ subunits with the peak elution times of standard proteins, we obtained information on the relative sizes and amounts of PFP complexes. When probing these elution time points with the PFPα antibody, both K0326Y and CM105 QPM 18-DAP extracts had a peak elution of 19 to 20 min, suggesting that the majority of PFPα in these extracts exists as a complex substantially larger than either the monomeric or dimeric form (Fig. 5, A and C). By contrast, the fractions containing PFPα in wild-type and o2 18-DAP extracts in both W64A and CM105 eluted later, peaking between 22 and 24 min (Fig. 5, A and C). This suggests that a smaller fraction of PFPα exists in tetrameric form in the wild type and o2. Additionally, no PFPα was detected in fractions later than 25 min in any genotype, suggesting that PFPα does not normally exist in its monomeric form. This may suggest that the majority of PFPα exists either as a homodimer or heterodimer with PFPβ in wild-type and o2 endosperm. In addition to a 20-min peak in CM105 QPM (Fig. 5C), there is a 23- to 24-min peak similar to o2 and the wild type, suggesting that PFPβ participates more in dimeric forms than in tetrameric forms at this early endosperm-filling stage (Fig. 5D).

Using the PFPβ subunit antibody, we detected the PFPβ subunit peaking at 20 to 21 min in QPM (Fig. 5B), which supports that PFPβ is part of a heterotetrameric complex in QPM endosperm. However, there is also a PFPβ-containing peak at 20 to 21 min in o2 and wild-type endosperm that does not correspond to the peak of PFPα elution (22–24 min; Fig. 5, A and C). This may suggest that in the presence of low amounts of PFPα, PFPβ may exist as a homotetramer, perhaps indicating that when induced, PFPα is able to displace PFPβ. PFPβ is detected weakly in the 23- to 24-min fractions and very weakly in the 25- to 26-min fractions, suggesting the presence of very little free monomeric PFPβ in any genotype. Interestingly, the 47-kD related protein described above is detected strongly in the 25- to 26-min fractions (Fig. 5B), suggesting that this protein does exist in the monomeric form and does not participate in the dimeric or tetrameric form.

We also fractionated native embryo extracts to determine if complexes form in this tissue (Fig. 6). Although both PFPα and PFPβ accumulate in embryo tissue, we did not detect differences in the size of the eluted fractions containing these subunits between the wild

Figure 4. PFP subunit accumulation in endosperm and embryo. A, PFPα protein gel blot of 12- and 18-DAP CM105 wild-type (WT), CM105 o2, and CM105 QPM endosperm extracts. B, PFPβ protein gel blot of 12- and 18-DAP CM105 wild-type, CM105 o2, and CM105 QPM endosperm extracts. C, PFPα protein gel blot of 18-DAP CM105 wild-type, CM105 o2, and CM105 QPM embryo extracts. D, PFPβ protein gel blot of 18-DAP endosperm extracts of representative vitreous, semivitreous, and opaque RILs derived from the W64A o2 × K0326Y QPM cross selected on the basis of phenotype rather than genotype. Kernel opacity phenotypes are shown below. Numbers below the panel refer to the RIL number whose genotype at chromosomes 1, 7, and 9 QTLs (Holding et al., 2011) follows the same nomenclature described Figure 3D legend, as follows: 164, BB AA AB; 208, AA BA AA; 175, AA BA BB; 176, AA BA BB; 185, BB BB BB; 194, BB BB BB. [See online article for color version of this figure.]

Figure 5. Size-exclusion chromatography analysis of PFP complex formation in maize endosperm. The panels show protein gel blots of SDS-PAGE of eluted fractions from HPLC separation of native endosperm extracts. Elution times of protein standards are shown in the key at bottom. Genotypes are shown on the left, and the developmental stages and the antibodies used are shown above each panel. WT, Wild type.
type, o2, or QPM, suggesting that activation of the PFP holoenzyme does not occur in the embryo (Fig. 6, A and B). Furthermore, rather than eluting at the predicted size of a tetramer, both subunits eluted largely between 22 and 24 min, suggesting that they exist in the dimeric form. Neither subunit appears to exist in the monomeric form (25–26 min). Interestingly, the predicted 47-kD protein, which is also recognized by the PFPβ antibody, does appear to exist as a monomeric form, as in endosperm (Fig. 6B).

**QPM Endosperm Has Reduced Forward PPDK Activity But Increased Forward PFP Activity**

The increased accumulation of PFPα transcript and protein prompted us to test PFP activity in wild-type, o2, and QPM developing endosperm (20 DAP) according to an established method (Theodorou et al., 1992). This showed that forward PFP activity is significantly lower in o2 compared with the wild type (P < 0.05), whereas the activity is almost 4-fold higher (P < 0.05) in QPM compared with o2 (Fig. 7). Since PFP is a reversible enzyme, and increase in PFPα abundance could cause an increased flux in the gluconeogenic direction, we also measured its reverse activity. Using the conditions described previously (Theodorou et al., 1992), reverse PFP activity was at least 100-fold lower than forward activity in all genotypes (data not shown), suggesting that in mid-kernel-filling endosperm, PFP function in the glycolytic direction may be most significant. However, since multiple factors that can affect relative forward and reverse flux in vivo can change very quickly, in vitro results with endosperm extracts are not sufficient for conclusive determination of in vivo flux.

In light of this possible increase in forward flux at an important rate-limiting step of glycolysis, we were interested to know if there are other glycolytic differences between the wild type, o2, and QPM (modified o2). There are two PPDK genes in maize (Sheen, 1991). cyPPDK1, through the existence of different promoters, encodes both a leaf-specific, chloroplast-targeted version and a cytosolic version, and cyPPDK2 encodes an exclusively cytosolic version (Sheen, 1991). cyPPDK1 has been reported to be positively regulated by O2 (Maddaloni et al., 1996). Consistent with this, semiquantitative RT-PCR showed a reduced level of expression of the cyPPDK1 gene in both o2 and QPM (modified o2; Supplemental Fig. S2). In contrast, we observed that cyPPDK2 expression is not markedly reduced in o2 compared with the wild type and, perhaps more significantly, that its expression is increased in QPM endosperm (Supplemental Fig. S2). Using a PPDK assay, we determined that forward PPDK activity is about 2-fold reduced in o2 and QPM compared with the wild type (P < 0.05 in both cases; Fig. 7). Interestingly, reverse PPDK activity is significantly reduced (P < 0.05) in QPM compared with o2. PFP and PPDK enzyme activities are expressed per milligram of protein. Soluble protein concentrations were highly stable between wild-type, o2, and QPM endosperm extracts, thus excluding the possibility that differences in the soluble protein concentration contribute significantly to the observed activity differences. Furthermore, we observed very similar results when activities were expressed per milligram fresh weight (data not shown).

The observed reduction in reverse PPDK activity, along with the forward PFP increase in QPM, would tend to shift net glycolytic flux in the forward direction. Reduced forward PPDK activity in o2 might result in reduced flow from phosphoenolpyruvate (PEP) to pyruvate (Fig. 1) and, therefore, reduced input into the TCA cycle. However, PEP may also be diverted through oxaloacetate and malate and into the TCA cycle. Interestingly, a gene for PEP carboxylase was found to be approximately 5-fold up-regulated in QPM over the wild type and o2 in the RNA-seq experiment, and an increase in expression was also observed in QPM and vitreous QPM RILs using semiquantitative RT-PCR (Supplemental Fig. S3).

**Amelioration of the Endosperm Stress Response in QPM**

We conducted an Illumina RNA-seq experiment to identify common differentially expressed genes in two independently bred QPM lines. In addition to genes whose expression increased, the experiment showed that the expression of many genes is reduced in QPM endosperm in comparison with o2 endosperm. As expected, the two different QPM lines tested exhibited substantial differences in the differentially expressed gene sets. However, we overlaid the data sets to find commonly down-regulated genes in QPM and found that heat shock proteins (Hsps) were well represented among the differentially expressed genes. These Hsps...
and other differentially expressed stress-related proteins are shown in Table I and suggest that modification of o2 endosperm is accompanied by stress amelioration. All classes of Hsps except Hsp60, including small Hsps (Hsp-30, Hsp-26), Hsp-70, Hsp-82, Hsp-90, and Hsp-101, are represented in this group. We used semiquantitative RT-PCR on a number of these genes to confirm this generalized Hsp down-regulation (Supplemental Fig. S4) and furthermore that the expression level is generally similar in wild-type and QPM endosperm but increased in o2 (Supplemental Fig. S4). Previous studies demonstrated the induction of stress-responsive genes in o2 endosperm compared with the wild type (Hunter et al., 2002), but these analyses did not include any QPM lines.

Of the 37 genes commonly down-regulated in both QPMs with respect to o2, eight were annotated as Hsps (Supplemental Table S1). We next compiled an estimate of the number of Hsp genes in the maize genome using two approaches (for details, see Supplemental Table S2). In the first, we collected Hsp annotation information from PlantGDB (http://www.plantgdb.org/) and identified Hsps in the B73 genome by homology. The second approach used keyword searches of MaizeGDB (http://www.maizegdb.org/). In this way, we identified 54 predicted nonredundant Hsps in the maize genome, and the gene identifiers and chromosome coordinates of these are shown in Supplemental Table S2. Therefore, our data show that approximately 15% of total Hsp genes are down-regulated in both QPM lines. If we compare the total number of Hsp genes (54) in the genome with an estimate of the total number of predicted maize genes (37,117; Eveland et al., 2010), eight Hsps out of 37 genes represents a very significant enrichment for Hsps in our differential gene set, as shown with a P value of $2.237 \times 10^{-16}$ by a hypergeometric test. As further confirmation of the significance of reduction in Hsp gene expression in QPM, we did not find any Hsp genes whose expression was increased in QPM.

PFP Is Induced by Stress in Maize Roots

To explore the possible link between the induction of ATP-requiring Hsp proteins and glycolytic modification, we subjected vegetative maize tissues to a variety of stresses, including heat stress, ER stress, and cold stress. Ten-day-old seedlings grown in tall sterile tissue culture boxes were incubated for 12 h at 42°C for heat shock, 4°C for cold shock, and 5 mM dithiothreitol (DTT) to induce ER stress. The DTT treatment was also combined with both heat and cold shock. We then used commercially available HSP antibodies and PFPα and PFPβ antibodies on western blots of protein extracts. Both PFPα and PFPβ accumulated at barely detectable levels in leaf tissue (data not shown). However, both PFP subunits accumulated at high levels in root tissues (Fig. 8). The level of PFPβ protein is not affected by the stress treatments, whereas heat stress, ER, and cold stress lead to increases in PFPα abundance. Heat and ER stress appear to be additive in their effects on PFPα induction (Fig. 8). Hsp90 is constitutively expressed in roots but is increased by heat stress. Hsp101 was only observed in roots after heat shock treatment. Hsp70 was detected constitutively but was increased by all treatments, especially heat shock. Antibodies to Hsp70 often detect a doublet in plant extracts, which likely corresponds to the ER lumen version (BiP) and a cytosolic version. Since the bottom band is induced by ER stress, the top band seen in lane 2 likely represents the cytosolic form (Fig. 8).

DISCUSSION

The increase in expression of molecular chaperones and other stress-related proteins in most opaque mutants including o2 has been documented previously (Hunter et al., 2002). In contrast to dominant floury2 (fl2), defective endosperm B30 (De-B30), and mucronate (Mc) mutants (Boston et al., 1991; Gillikin et al., 1997; Kim et al., 2004, 2006), which accumulate abnormal zeins, the cause of endosperm stress in most opaque...
Table I. Heat shock proteins and other stress-related proteins that are commonly down-regulated in K0326Y QPM and CM105 QPM with respect to W64Ao2 and CM105o2

Boldface columns represent actual fold reduction in each QPM line compared with o2 based on the relative numbers of reads for each gene (see “Materials and Methods”). RT-PCR analysis (Supplemental Fig. S4) showed increased expression in o2 compared with both the wild type and QPM (italicized text), and genes for which RT-PCR could not detect differences are shown with underlined text. Genes not tested with RT-PCR are shown without formatting.

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<th>Gene Identifier</th>
<th>K0326Y QPM</th>
<th>P</th>
<th>CM105QPM</th>
<th>P</th>
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<th>Gene Start</th>
<th>Gene End</th>
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mutants is unknown. Although unfolded protein response (UPR) markers are only slightly elevated in o2 endosperm (Hunter et al., 2002), the increase in Hsps and other stress-related proteins does suggest cellular stress. Since zein accumulation is reduced, many nonzein proteins are increased to higher than normal levels as a result of a rebalancing of the seed proteome. Abnormally high accumulation of certain nonzein proteins may be the source of the observed cellular stress in o2, since most nonzeins would not be retained within ER protein bodies and may thus overwhelm the downstream secretory system. o2, De-B30, and fl2 all have a low-zein phenotype, and it has been assumed that altered zein protein body formation is the primary cause of opacity and that cellular stress is only a side effect of this. However, other mutants such as o1 show no low-zein or elevated nonzein phenotype, yet o1 has increased expression of a number of stress-related genes and chaperone genes (Hunter et al., 2002), inviting the suggestion that the stress response itself may be at least partially responsible for the opacity in o1 and, perhaps, other opaque endosperm mutants.

The above hypothesis is compatible with the lowering of expression of stress response genes in QPM (modified o2) to wild-type expression levels and possibly suggests that o2 modifiers work by directly ameliorating the stress response. The RNA-seq experiment identified a group of 37 genes decreased in expression in both independent QPM lines in relation to o2. Among this gene set, eight genes (22%) were Hsps, and others were annotated as Hsp transcription factors, Hsp-binding proteins, and other stress-related genes (Table I). These results may suggest that the modification of o2 endosperm is accompanied by, or even driven by, stress amelioration. Most classes of chaperones/
How could the stress response be ameliorated in QPM? We have described our results showing the substantial increase in the abundance of the PFPα regulatory subunit in QPM and its reduction in o2 endosperm. In contrast, PFPα abundance is not increased in QPM embryo. Furthermore, we have shown that while QPM endosperm accumulates a PFP complex with the size of the previously predicted active tetramer, wild-type and o2 endosperm accumulate much less of the predicted active tetramer. Furthermore, tetramer formation does not occur in QPM embryo, supporting the suggestion that PFP induction is specifically involved in ameliorating the endosperm defect. Our in vitro assay results indicate that forward PFP activity is about 4-fold increased in QPM relative to o2 endosperm, consistent with the formation of the predicted active tetramer. The function of PFP in plants is not certain, although several roles related to the modulation of respiration have been proposed (Plaxton and Podesta, 2006). Most evidence suggests that PFP functions as an adaptive enzyme that enables plants to survive certain stresses by inducing PPI-dependent bypasses in order to conserve diminished ATP reserves (Plaxton and Podesta, 2006). We have shown in this paper that PFPα protein is induced in response to heat shock, UPR, and cold shock in maize root tissues. This supports the idea that PFP participates in glycolytic modification to meet the additional ATP demand created by a generalized Hsp induction. PFP may be functioning in endosperm modification by adopting a similar adaptive role. If nonzein proteins accumulating to unnaturally high levels in o2 place an increased demand on the basal protein-folding machinery, this could create a drain on ATP levels. If ATP became limiting, this could cause the folding machinery to become overwhelmed, thus leading to increased expression of Hsps. Also, although PPDK functions in the forward and reverse directions, our in vitro assay results suggest a significant reduction in its forward activity in o2. This may contribute to a reduced glycolytic flux and ATP synthesis. Furthermore, although we observed a reduced expression of cyPPDK1 in both o2 and QPM endosperm (Supplemental Fig. S2), consistent with its regulation by O2 (Maddaloni et al., 1996), we detected increased expression of cyPPDK2 in QPM. In developing QPM endosperm, increased PFP and possibly cyPPDK2 activity could conserve ATP levels by enhancing non-ATP-dependent glycolytic flux. In this case, ATP may not become limiting, the basal HSP machinery would be able to cope with the increased nonzein protein accumulation, and the stress response would not be invoked.

An alternative explanation for the increase in PFP activity in QPM could be that it serves to prevent PPI from accumulating. We measured approximately a 2-fold reduction in forward PPDK activity in both o2 and QPM, and one possible consequence of this would be an increase in PPI. This might tend to inhibit the forward activity of ADP-Glc pyrophosphorylase and thus reduce starch synthesis in o2 and, conceivably, contribute to the kernel phenotype. However, PPI assays indicated that PPI does not increase in o2 or QPM endosperm relative to the wild type (data not shown), which does not support the above hypothesis. Thus, the stability of PPI in plant tissues supports the suggestion that it serves as an alternative energy currency when ATP is limited. Reduced forward flow at the PPDK step in o2 could restrict flow to pyruvate and, thus, ATP production through reduced flux to the TCA cycle. To some extent, QPM endosperm may be able to compensate for this blockage by increasing PEP carboxylase activity, consistent with its increased transcription, providing a bypass route into the TCA cycle. However, since the central endosperm environment becomes hypoxic during development (Rolletschek et al., 2005), glycolysis alone may be increasingly important for ATP production as the TCA cycle and oxidative phosphorylation slow down. A reduction in forward PPDK activity in o2 endosperm could shift the equilibrium of this reaction toward PEP. This may have a negative effect on ATP production through glycolysis, because PEP is a potent feedback inhibitor of ATP-dependent phosphofructokinase in virtually all plants tested (Plaxton, 1996). Since the PFP enzyme is not negatively affected by PEP, this lends further support to the suggestion that its up-regulation in QPM provides a way to maintain glycolytic ATP production. Furthermore, since PPI acts to reverse the negative inhibitory effect of PEP on phosphofructokinase, PPI up-regulation would be predicted to have a further positive effect on glycolysis, since it produces PPI in the forward direction (Plaxton, 1996).

An increased ratio of nonzein to zein proteins is largely responsible for improved amino acid balance in o2 (Mertz et al., 1964; Moro et al., 1996), and this increase is equally apparent in modified o2 (QPM) despite the increased γ-zein accumulation. If PEP carboxylase activity is increased in QPM, this could also impact amino acid biosynthesis, since oxaloacetate is a precursor to several amino acids, including Lys (Fig. 1). Despite the dramatic changes in the proportions of zeins and nonzeins, at the endosperm level total protein content remains relatively constant between normal and o2 kernels (Supplemental Fig. S5; Mertz et al., 1964), suggesting that endosperm protein content is determined by the amount of available nitrogen. This may suggest that the changes we have described in PFP and PPDK activities are not primarily driven by a need for increased amino acid production to compensate a general deficit in protein synthesis. On the other hand, an increase in Lys-containing nonzein proteins may indeed require additional flux through oxaloacetate and the Asp pathway (Fig. 1), and it is possible that differences exist between o2 and QPM at this step. To further investigate glycolytic alterations in o2 and QPM and the significance of ATP and PPI levels in developing endosperm, as well as flux to amino acid synthesis through oxaloacetate, we are conducting complete metabolic profiling of central carbon metabolites in early, mid, and late endosperm-filling stages of wild-type, o2, and QPM kernels. To better understand the significance of PFP and PPDK to endosperm maturation, we are preparing transgenic lines with endosperm-specific over-
expression as well as RNA interference suppression for both genes. 

During our investigation of the differentially expressed PFPα genes, we identified two highly similar but slightly different-length transcripts that share the same predicted ORF but that are transcribed from different genes. The longer of the two transcripts results from a gene with multiple introns and has a 105-bp exon that is absent in the shorter transcript. By contrast, the genomic sequence of the shorter gene is identical in length and sequence to its cDNA; it contains no introns. This indicates that this gene is derived from a reverse-transcribed copy of a spliced mRNA that lost the 105-bp exon and underwent a reinsertion event into the genome. This phenomenon, called repositioning, is well known in animals and is a significant evolutionary driving force (Brosius, 1991). Most retrogenes are non-functional pseudogenes, since they do not have promoters. However, if a retrogene happens to insert into a region capable of regulation, not only can it be expressed but it can be renovated with novel introns. This has recently been demonstrated in plants (Zhu et al., 2009), although much remains to be learned about the mechanism and its functional significance. The PFPα retrogene may be functional, since it is transcribed, although its function in relation to the longer, inducible retrogene may be functional, since it is transcribed, a sr e c e n l yb e e nd e m o n s t r a t e di np l a n t s( Z h ue ta l . , 2008 )

Nucleic Acid Extraction and RT-PCR

Genomic DNA for PCR was extracted and diluted from whole frozen developing kernels (Holding et al., 2008). Total RNA extraction from 18-DAP endosperm and embryo tissues, DNase treatment, cDNA synthesis, and amplification were all performed as described previously (Holding et al., 2008, 2011). For qRT-PCR, primers entirely within the 105-bp region (not present in the retrogene; PFP-105R-F [5'-CGAAGCTCTATCTGGCAAGAAC-3'] and PFP-105R-R [5'-GCGAGCGTCTGCATTCTGACCG-3']) and primers common to both PFPα and PFPβ retrogene (PFP-RT-F2 [5'-GATCCCTATGCGGAAGAAC-3'] and PFP-RT-R2 [5'-TCCACTAATTGCGGAAGACA-3']) were used. 

immunoblot Analysis

Proteins were extracted from frozen 18-DAP endosperm and embryo tissues as well as leaf and root tissue using lysis buffer (10% [v/v] glycerol, 50 mM Tris, pH 8, 50 mM NaCl, 5 mM MgCl₂, 0.1% [v/v] Nonidet P-40, 2 mM DTT, and 1 mM phenylmethylsulfonyl fluoride) and stored at −80°C. Ten- to 20-μg protein samples were loaded on 12.5% SDS-PAGE gels, run using the Mini-protein system (Bio-Rad), and blotted onto nitrocellulose using the Mini-transblot system (Bio-Rad) according to standard protocols. Blots were blocked in TBST (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.05% [v/v] Tween 20) with 3% milk for 1 h at room temperature. Affinity-purified PFPα and PFPβ peptide antibodies were acquired from GenScript and used at 1:4,000 dilution in TBST with 3% (w/v) milk for 2 h at room temperature. After extensive washes in TBST, secondary antibody (goat anti-rabbit IgG conjugated to horseradish peroxidase [Pierce]) was applied at 1:10,000 dilution in TBST with 3% (w/v) milk for 2 h at room temperature. After extensive washes in TBST, secondary antibody was detected with the Super Signal West Pico chemiluminescent substrate kit (Pierce) according to the manufacturer’s instructions.

Size-Exclusion Chromatography

The 32 karat soybean was used to operate a Beckman Coulter system gold 126N solvent module Beckman SC100, size-exclusion chromatography system. Gel-filtration standards (Bio-Rad) were used to calibrate a Superose 12/300 GL column (GE Healthcare) with phosphate-buffered saline buffer. Under our conditions, a flow rate of 0.5 mL min⁻¹, each size standard eluted over a 4- to 5-min range. The peak elution times for each size standard were as follows: 670-kDa thyroglobulin (bovine), 17 min; 158-kDa γ-globulin (bovine), 24 min; 44-kDa ovalbumin (chicken), 27 min; 17-kDa myoglobin (horse), 31 min; 1.35-kDa vitamin B12, 40 min. Samples of nondenatured protein (250 μg) in 250-μL volumes were loaded. The flow rate was set at 0.5 mL min⁻¹, and 0.5-mL fractions were collected every minute. Fractions (250 μL) from the indicated time points were precipitated with 1 mL of acetone at −20°C overnight, centrifuged at 16,000g for 15 min at 4°C, resuspended in 25 μL of SDS loading buffer and 10-μL volumes were separated on SDS-PAGE gels and the presence of denatured subunits in native elution time points were determined by western detection.

Enzyme Assays

Kernels from ears that had been frozen in liquid nitrogen were partially defrosted for embryo removal and immediately refrozen in liquid nitrogen. Fresh weight measurement was taken before homogenizing endosperms to a fine powder. For the PPDK assay, protein extraction buffer (1 mL) was added containing 50 mM Tris (pH 8.0), 5% (v/v) glycerol, 50 mM NaCl, 5 mM MgCl₂, 0.1% (v/v) Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 0.5 mM DTT, and protein inhibitor cocktail. Samples were then incubated at 4°C for 30 min on a rotating wheel at 10 rpm. Extracts were clarified by centrifugation at 4°C at 16,000g for 15 min. All extracts were kept at 4°C during analysis and stored at

PFP Induction in Quality Protein Maize Endosperm

GTGCGACTGAAGTGGTGTG-3') and PFP-intron1-F (5'-CAACGACTCTACGTTGAAAC-3') and PFP-intron1-R (5'-GATCGCCTGCTGGACAGA-3'). Part of the very large second intron was obtained through the primers pairs PFP-215-F (5'-CACTTCCAGAAAGAGACCAAGA-3')/PFP-intron2-R (5'-GCCCTGCCTATCTGGTCTAA-3') and PFP-intron2-F (5'-CCTCGGGCTTGCTGTCCTA-3')/PFP-215-R (5'-CAATGCTGCCCTACCAGAACA-3'). The middle part was inferred through the maize (Zea mays) genomic database.
~80°C if needed for further assays. PDPK was assayed in the forward direction (pyruvate formation) using an established method (Wang et al., 2008). PDPK activity was measured by a coupling reaction of NADH oxidation through PEP carboxylase and malate dehydrogenase. The assay buffer consisted of 100 mM HEPES-NaOH (pH 8.0), 15 mM MgCl₂, 0.15 mM EDTA, 5 mM NaHCO₃, 0.3 mM NADH, 5 mM NaH₂Cit, 2.5 mM K₂HPO₄, 5 mM DTT, 1 mM Glc-6-P, 1.5 mM ATP, and 10.5 units of malate dehydrogenase. The reaction was initiated by the addition of pyruvate to 1.25 mM final concentration and 3 units of purified maize PEP carboxylase (Bio-Research Products). PDPK was also assayed in the reverse direction (PEP formation) according to an established method (Andrews and Hatch, 1969). Activity was determined by measuring pyruvate production in the presence of lactate dehydrogenase. Assay buffer consisted of 50 mM Tris-HCl buffer (pH 8.3), 6 mM MgSO₄, 10 mM DTT, 0.15 mM NADH, 1 mM PEP, and 1 mM AMP with the addition of 6 units of lactate dehydrogenase and 1 mM Pi to stimulate activity in a 1-mL total volume. The rate of activity was measured at 340 nm and 30°C for all enzymatic assays.

For the PPF assays, frozen ground endosperm tissue was mixed and incubated with the same protein lysis buffer as for the PDPK assays. PPF was assayed in the forward and reverse directions essentially as described previously (Theodorou et al., 1992). Forward PPF activity was assayed by following the oxidation of NADH in 50 mM Tris-HCl (pH 7.5) containing 5 mM Fru-6-P, 10.5 units of malate dehydrogenase, and 1 unit of glycerol-3-phosphate isomerase, and 1 unit of glyceraldehyde phosphate dehydrogenase.

Assays were initiated by the addition of PPF, and PPF activity was monitored for approximately 3 min. Fru-2,6-bisP (2 μM) was then added to stimulate PPF activity and monitored for approximately 4 min. Reverse PPF activity was assayed following the reduction of NADP⁺ in the following assay buffer: 50 mM Tris-HCl (pH 7.5), 5 mM MgCl₂, 0.5 mM Fru-1,6-bisP, and 1 mM NADP⁺. Assays were initiated by the addition of the extract, NaPi (5 mM; pH 7.5), 2 units of phosphoglucoisomerase, and 1 unit of Glc-6-P dehydrogenase and monitored for approximately 4 min. All PPF activity was assayed at 25°C in a 1.0-mL final volume.

Illumina RNA-seq

RNA-seq was used to compare the following six genotypes: W64A wild type, W64A opaque2, K0326Y QPM, CM105 wild type, CM105s2, and CM105 QPM. For each genotype, a pool from three 18-DAP ears, eight dissected endosperms per ear, was used for cDNA library preparation, and 51-bp reads were obtained in Illumina sequencing reactions. By overlaying the sets of differentially expressed genes from the two different QPMs, we identified common differentially expressed genes including those down-regulated in QPM with respect to K0326Y, N. By comparing the expression of the maize immunoglobulin binding protein homolog b-70 in three stress-related genes from Table 1.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. qRT-PCR analysis of PFPa expression in CM105 wild type, CM105 opaque2, and CM105 QPM.

Supplemental Figure S2. Semi-qRT-PCR analysis of cyPFPK1 and cyPFPR2 expression in W64A wild type, W64A opaque2, and K0326Y QPM.

Supplemental Figure S3. Semi-qRT-PCR analysis of phosphoenolpyruvate carboxylase expression in W64A wild type, W64A opaque2, K0326Y QPM, and K0326Y RILs.

Supplemental Figure S4. Semi-qRT-PCR verification of Hsp and other stress-related genes from Table 1.

Supplemental Figure S5. Total protein content in maize endosperm.

Supplemental Table S1. Common down-regulated genes in both K0326Y QPM and CM105 QPM from RNA-seq analysis.

Supplemental Table S2. Heat shock protein genes from the B73 maize genome.

Note Added in Proof

The GenBank accession numbers for PFPa mRNA and the PFPa retrogene mRNA described in this manuscript are JQ522972 and JQ522973, respectively.

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