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## *Analyses of* o2, o7, *and* o2o7 *mutations on amino acid metabolisms in maize endosperm by transcript profiling**

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Our long-term goal is comprehensive dissection of the molecular mechanisms underlying endosperm development and metabolisms in maize kernels. In this context, genetics has played an important role by discovering a series of opaque endosperm mutants and demonstrating their effects on genes mediating zein deposition (see Motto et al., Maydica 54: 321-342, 2009). For example, the recessive mutation *opaque-2* (*o2*) and *opaque-7* (*o7*) induce a specific decrease in the accumulation of 22- and 19-kDa α-zeins, respectively.

The *o2* mutation has been widely studied at the genetic, biochemical, and molecular levels. It was shown that *O2* encodes a basic leucine zipper (bZIP) transcriptional regulator that is specifically expressed in the endosperm; it also activates the expression of 22-kDa α-zein and 15-kDa β-zein genes by interacting with the TC-CACGT(a/c)R(a/t) and GATGYRRTGG sequences of their promoters, therefore displaying a broad binding specificity and recognizing a variety of target sites in several distinct genes (Gavazzi et al., Plant Physiol. 145: 933-945, 2007). *O2* also regulates directly or indirectly a number of other non-storage protein genes, including *b-32*, encoding a type I ribosome-inactivating protein, *cyPPDK1*, one of the two cytosolic isoforms of the pyruvate orthophosphate dikinase gene, and b-70, encoding a heat shock protein 70 analogue, possibly acting as a chaperonin during protein body formation (cf. Motto et al., Maydica 54: 321-342, 2009). *O2,* furthermore, regulates the levels of lysine-ketoglutarate reductase (Brochetto-Braga et al., Plant Physiol., 98:1139-1147, 1992 ) and aspartate kinase1 (Azevedo et al., Phytochemistry, 46**:**395-419,1997).These broad effects suggest that *O2* plays an important role in the developing grain as a coordinator of the expression of genes controlling storage protein, and N and C metabolisms.

Although the molecular basis of *o7* mutation is yet unknown, evidence indicates that this mutation, in addition to repressing the lower molecular weight α-zeins*,* drastically affects the development of maize endosperm due to a reduction in starch content. Moreover, the high content in *o7* endosperms of non-protein N has suggested the existence in *o7* of a block in the synthetic

route leading to proteins similar to that observed for the starch modifying gene *shrunken4* (Motto et al., Maydica 54: 321-342, 2009).

To advance our understanding of the nature of the *o2* and *o7* mutations in affecting amino acid metabolisms, we used genome-wide analyses of gene expression profiles during kernel development. Specifically, for this study we have used mRNA transcripts from A69Y+ and from the nearly isogenic *o2* and *o7* mutants, and for the double mutant combination *o2o7,* collected from endosperms at 14 days after pollination. Additionally, the profile of endosperm transcripts was obtained with the Zeastar Unigene set, based on the sequence information of >7,200 maize genes, mainly derived from maize endosperm and covering a wide range of metabolic pathways and cellular and physiological processes

Our study indicated that several ESTs homologous to enzymes involved in amino acid synthesis were differentially expressed in the *o2*, *o7*, and *o2o7* endosperms. In particular, ESTs homologous to enzymes involved in tryptophan synthesis were affected in *o2* endosperm. Tryptophan synthase (EC 4.2.1.20) homologues showed a significant reduction of expression in *o2* endosperms, while anthranilate phosphoribosyl transferase (EC 2.4.2.18) and anthranilate synthase (EC 4.1.3.27) homologous ESTs were found to be differentially expressed in all three mutant backgrounds. The former showed a significant reduction of its expression level, while the latter appeared up-regulated by 50%.

ESTs homologous to phosphoglycerate dehydrogenase (EC 1.1.195), cysteine synthase (EC 2.5.1.47), methionine synthase (EC 2.1.1.14), S-adenosylmethionine synthetase (EC 2.5.1.6), and a methyl transferase (EC 2.1.1.37), all enzymes involved in the Ser, Gly, Cys, and Met pathways were negatively affected in the *o2* endosperm. However, neither of these showed a significantly altered expression level in the *o7* and *o2o7* endosperms. Finally, the Ile, Val and Leu pathways were affected in all three lines. ESTs homologous to acetolactate synthase (EC 2.2.1.6) and ketolacid reductoisomerase (EC 1.1.1.86), and involved in the biosynthesis of these amino acids were significantly reduced in expression in all three backgrounds, while leucine dehydrogenase (EC 1.4.1.9) was significantly different from wild-type only in the o7 endosperms.

In conclusion, the current study indicated that the transcription levels of various genes encoding key enzymes involved in amino acids were significantly affected in the *o2* mutant. *O7* regulates the expression of some genes of the amino acids biosynthesis, but only in few cases the mRNAs affected are the same that are up- or down-regulated in the *o2* mutant, suggesting that the *O2* and *O7* factors act on specific target genes. Among the pathways affected by *o2* and *o7* mutants are those leading to the synthesis of the aromatic (Phe, Trp, and Tyr), Asp-derived, and branched chain amino acids (BCAA). These pathways are deeply interconnected both in terms of C precursor supply and of allosteric interactions (Curien et al., Plant Physiol. Biochem., 46:325-339, 2008). A complex interplay of regulators controls the metabolic flow through the aromatic, Asp and BCAA-pathways, which includes feedback inhibitors of regulatory enzymes (Galili and Höfgen,

Metabolic Eng., 4:3-11, 2002). Furthermore, alterations in enzymes affecting amino acid metabolism have been shown to have pleiotropic effects on free amino acid levels in plant tissues. For example, it was found that a mutation in a key enzyme in the Asp-pathway, a feedback-insensitive aspartate kinase mutant in tobacco, not only has a higher level of amino acids derived from the Asp pathway, but other pathways as well (Frankard et al., Plant Physiol., 99: 1285-1293, 1992). In addition, it was reported that the alteration of Trp and Tyr levels in transgenic tobacco leaves affects the level of Trp, as well as the aliphatic amino acids Met, Val, and Leu (Guillet et al., Plant Physiol., 122:933-943, 2000).

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