ABSTRACT

The Role of General Control Non-Derepressible 2 in Maize Growth, Development and Stress Response

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Maize is among the most important food sources in the world. However, maize protein is not nutritionally balanced, limiting its value. As a result of climate change, frequent heat and drought stresses negatively affect maize yield. Therefore, it is critical to improve maize nutritional value, yield and performance under stress conditions to sustain global food security. General control non-derepressible-2 (GCN2) plays an important role in cellular responses to amino acid starvation. During amino acid starvation, GCN2 phosphorylates the α subunit of eukaryotic translation initiation factor-2 (eIF2), which enhances the translation of the transcription factor GCN4 by overcoming the inhibitory effect of upstream open reading frames (uORFs). This results in increased expression of many amino acid synthesis genes, and is known as general amino acid control. In this study, we first investigated the pleiotropic effects of maize *opaque2 (o2)* mutation. The *o2* mutant was discovered to have enhanced essential amino acid lysine content, demonstrating nutritional superiority. We found elevated protein accumulations that could partially explain the high lysine content and altered gene expression associated with the increased

insect and fungal susceptibility and the brittle endosperm texture of *o2*. Next, we showed that maize GCN2 kinase phosphorylated eIF2α in response to amino acid starvation in maize endosperm. It was associated with an increase of O2 protein accumulation but no alteration of *O2* transcript was detected, indicating that the regulation of O2 was translational and that O2 could be a maize GCN4 ortholog. We then tested the role of GCN2 in other stress situations, such as drought. The *gcn2* mutant showed increased tolerance to drought compared to wild type and had a higher level of steady state abscisic acid (ABA). RNA-Seq analysis indicated candidate genes responsible for the increased tolerance but further analysis was necessary for candidate genes involved in elevated ABA abundance. Finally, we screened for proteins involved in the localization of the 27 kDa γ-zein, which is important for the protein body formation in maize endosperm, and found that maize eIF5A was associated with γ-zein mRNA and an actin rich cytoskeleton, indicating the possible role of eIF5A in localizing γ-zein.

The Role of General Control Non-Derepressible 2 in Maize Growth, Development and Stress Response

by

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

| | |
|---|---|
| AAA-ATPase | Proteosome regulatory subunit AAA-ATPase |
| AAO | Abscisic acid aldehyde oxidase |
| ABA | Abscisic acid |
| ACC | 1-aminocyclopropane-1-carboxylic acid |
| AFLP | Amplified fragment length polymorphism |
| AGP | Arabinogalactan protein |
| ATF4 | Activation transcription factor 4 |
| BAP2 | Basal layer antifungal protein2 |
| BEI | Starch branching enzyme I |
| BEIIa | Starch branching enzyme IIa |
| BEIIb | Starch branching enzyme IIb |
| BGAF | Beta-glucosidase aggregating factor |
| CBL | Calcineurin B-like protein |
| CIPK | Calcineurin B-like protein ineracting protein kinase |
| CI-1B | Subtilisin-chymotrypsin inhibitor CI-1B |
| CTAB | Cetyltrimethylammonium bromide |
| DAP | Days after pollination |
| DHN | Dehydrin |
| DSC | Differential scanning calorimetry |
| EF1A | Translation elongation factor 1 alpha |
| eIF2α | Eukaryotic translation initiation factor 2 alpha subunit |

| | |
|---|---|
| eIF2B | Eukaryotic translation initiation factor 2B |
| eIF4E | Eukaryotic translation initiation factor 4E |
| eIF4G | Eukaryotic translation initiation factor 4G |
| ER | Endoplasmic reticulum |
| ERO1 | ER Membrane-Localized Oxidoreductase 1 |
| FKPM | Fragments per kilobase of exon per million fragments mapped |
| GAAC | General amino acid control |
| GAPDH | glyceraldehyde-3-posphate dehydrogenase |
| GBSSI | Granule-bound starch synthase I |
| GC-MS | Gas chromatography-mass spectrometry |
| GCN2 | General control non-derepressible 2 |
| GCN4 | General control non-derepressible 4 |
| GDP | Guanosine diphosphate |
| GDSL | Glycine–aspartic acid–serine-leucine |
| GO | Gene ontology |
| GTP | Guanosine triphosphate |
| HSP3 | 17.4-kDa Class I heat shock protein 3 |
| IF5A | Translation initiation factor 5A |
| LEA | Late embryogenesis abundance protein |
| LKR-SDH1 | Lysine-ketoglutarate reductase/saccharopine dehydrogenase1 |
| MAPK | Mitogen-activated protein kinase |
| MCSU | Molybdopterin cofactor sulfurase |

| | |
|---|---|
| MEE21 | Maternal effect embryo arrest 21 |
| mORF | Major open reading frame |
| *Mu* | *Mutator* |
| NAC | NAM, ATAF, and CUC transcription factor |
| NCED | Nine-*cis*-epoxycarotenoid dioxygenase |
| O2 | Opaque2 |
| PB | Protein body |
| PEG | Polyethylene glycol |
| PDI | Protein disulfide isomerase |
| PGK | Cytosolic phosphoglycerate kinase |
| PP2C | Protein phosphatase 2C |
| qRT-PCR | Quantitative real-time polymerase chain reaction |
| RACE | Rapid amplification of cDNA ends |
| RBOHB | Respiratory burst oxidase protein homolog B |
| RIP | Ribosome-inactivating protein |
| RNAi | RNA interference |
| ROS | Reactive oxygen species |
| RRB1 | Retinoblastoma-related protein 1 |
| RT | Room temperature |
| RWD | RING-finger, WD40, DEAD-box helicase |
| S6RP | Ribosomal protein S6 |
| SDH1 | Sorbitol dehydrogenase 1 |
| SDR | Short-chain dehydrogenase/reductase |

| | |
|---|---|
| SSIIa | Starch synthase IIa |
| SSIII | Starch synthase III |
| TC | Ternary complex |
| TSJT1 | Stem-specific protein |
| TA1 | Tryptophan aminotransferase |
| TIM | Triose phosphate isomerase |
| TUSC | Trait utility system for corn |
| uORF | Upstream open reading frame |
| UTR | Untranslated region |
| UPR | Unfolded protein response |
| VP14 | Viviparous 14 |
| ZEP | Zeaxanthin epoxidase |
| Zpu1 | Pullulanase-type starch debranching enzyme1 |

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your unconditional love and for believing in me from the beginning. I hope you are proud of me.

# DEDICATION

To My Parents, Yan and Jianguo

# CHAPTER ONE

# Introduction

## *Maize*

Maize is one of the most important cereal crops in the world. About 10% of the maize produced in the US is for human consumption and 40% for livestock feed (Solomon, 2007). In less developed regions such as southern Africa, maize accounts for over 50% of the calories intake in local diets (McCann, 2001). Because of the rapid growth of human population, the demand for maize and other cereal grains will continue to increase (Gibbon and Larkins, 2005). Over the past decades, global warming, deforestation and urbanization have caused increased frequency of heat, drought and flood stresses, which negatively affected crop yields, especially at low latitudes (Barber, 2001; UNECA, 2011; Zhang et al., 2005). The ability of maize to maintain nutritional quality and high yields under heat and water stress is critical to sustain global food security, especially as growing human population demands an increase in food staples (Ortiz et al., 2008).

## *Zeins and Nutritional Imbalance*

Despite being a major food source, maize does not provide a nutritionally balanced source of protein. Over half of the proteins in maize endosperms are prolamin storage proteins, which are called zeins. Zein proteins belong to a multigene family in maize that consists of four structurally distinct types: α-, β-, γ- and δ-zeins, with α-zeins being the most abundant and γ-zeins regulating the organization of α-zeins during protein body (PB) formation (Achberger et al., 2012; Coleman and Larkins, 1999; Coleman et al., 1995;

Shewry and Halford, 2002). Zeins are highly hydrophobic, thus insoluble in water but soluble in reagents such as high percentage aqueous ethanol (Esen et al., 1987; Rees and Singer, 1956). Zeins are rich in proline and glutamine but lack several essential amino acids that cannot be synthesized by monogastric animals, such as lysine and tryptophan (Gibbon and Larkins, 2005). Therefore, monogastric animals need to obtain the essential amino acids from their diet and usually suffer from symptoms caused by lysine deficiency when consuming maize as the only protein source (Harris et al., 1943). Lysine deficiency interferes with the biosynthesis of carnitine and has negative impacts on growth, bone formation, mitotic activities in male reproductive organs and the number of blood cells in rats (Cox and Hoppel, 1973; Harris et al., 1943). To achieve nutritional balances, farmers traditionally apply maize-soybean diet or add artificial lysine to the maize only diet, which increases the feed cost, and eventually increases the prices of meat. In less developed countries where maize is the most common food source, it is often the sole source for carbohydrate and protein intake due to the socioeconomic status (Williams, 1933). As a result, malnutrition is a frequent cause of disease and death in the third world countries, with protein deficiency being the most severe problem (Latham 1997).

### *Opaque 2*

Extensive efforts have been made to identify maize mutants with increased lysine contents. In the 1960s, two maize mutants, *opaque 2* (*o2*) and *floury 2*, were discovered to have elevated lysine content in the mutant endosperm (Mertz et al., 1964; Mertz et al., 1965). The maize *o2* mutant has a substantially higher lysine content in the endosperm (+69%) and significantly reduced level of zein protein accumulation (Mertz et al., 1964). The *o2* mutant has been shown to have superior nutritional value compared to the wild type

(Clark et al., 1967; Cromwell et al., 1967). To obtain the same amount of biologically valuable proteins through consuming a maize only diet, only half the amount of *o2* kernels by weight is needed compared to the wild type kernels (Krivanek et al., 2007). Therefore, *o2* has been intensively studied in order to improve the nutritional quality of maize as human food and animal feed. However, *o2* kernels are soft and starchy in texture, which limits the agronomic production due to the brittleness and the susceptibility to insect and fungal attack (Crow and Kermicle, 2002; Gibbon and Larkins, 2005).

The *O2* gene has been cloned and encodes a b-zip transcription factor that is expressed specifically in the maize endosperm. O2 activates the transcription of zeins through direct interaction with the zein promoter elements (Schmidt et al., 1990). Thus in the *o2* mutant, the expression of zeins is significantly reduced, with 22 kDa α-zein being the main target of O2 regulation (Schmidt et al., 1990; Schmidt et al.). The *o2* mutation has pleiotropic effects by altering the expression levels of many other target genes, resulting in increased essential amino acid contents, altered kernel texture and decreased defense as mentioned above. Several transcriptomic and proteomic studies have been carried out to understand the underlying mechanism of the pleiotropic effects of *o2* mutation (Damerval and Le Guilloux, 1998; Frizzi et al., 2010; Frizzi et al., 2008; Hunter et al., 2002; Jia et al., 2007). These studies have consistent observations that the genes involved in endoplasm reticulum (ER) stress responses and glycolytic pathway are up regulated in the *o2* mutant. However, these results alone still could not explain some of the intriguing phenotypes of *o2*.

## *General Amino Acid Control*

In yeast (*Saccharomyces cerevisiae*), many amino acid biosynthesis genes are regulated under a common control mechanism. When one or several amino acids become limiting, the expression levels of these genes increase in response to amino acid starvation, causing a derepression of amino acid biosynthesis gene expression (Hinnebusch, 1988; Hinnebusch and Fink, 1983). For example, arginine starvation induces the derepression of arginine synthesis enzyme as well as the biosynthesis of tryptophan, histidine, lysine, isoleucine-valine and leucine (Delforge et al., 1975). Histidine starvation leads to derepression of biosynthesis of histidine as well as arginine, tryptophan and lysine (Delforge et al., 1975; Hinnebusch and Fink, 1983). In general, more than 30 amino acid biosynthesis enzymes representing 12 different pathways are induced in response to amino acid starvation (Hinnebusch, 1986, 2005). This general regulation of amino acid biosynthesis is known as general amino acid control (GAAC). In yeast, the GAAC response is stimulated through translational regulation of the yeast b-zip transcription factor general control non-derepressible 4 (GCN4) (Hinnebusch, 1984, 2005), which is discussed later in detail.

## *GCN2 Signaling Pathway*

### *Eukaryotic Translation Initiation*

Translation initiation is an essential control point for protein synthesis in all eukaryotes. Eukaryotic initiation factor 2 (eIF2) is a heterotrimer (subunits α, β and γ) that can bind to either guanosine triphosphate (GTP) or guanosine diphosphate (GDP). When bound to GTP, it is able to bind Met-tRNA to the P site of the 40S small ribosomal subunit.

The attachment of the (eIF2.GTP.Met-tRNA) ternary complex (TC) to the 40S subunit forms the 43S ribosomal subunit, binds to the mRNA and begins to scan the 5' untranslated region (UTR). At the AUG start codon, GTP is hydrolyzed to GDP, releasing eIF2 from the ribosomal subunit in a stable inactive binary complex with GDP (Kimball, 1999) (Figure 1.1). The 60S ribosomal subunit joins the rest of the initiation apparatus to form the 80S initiation complex and translation continues (Kozak, 1983). The five-subunit guanine-nucleotide exchange factor eukaryotic initiation factor 2B (eIF2B) is necessary to recycle eIF2-GDP to eIF2-GTP (Sonenberg and Dever, 2003). This recycling process is the major target of translational control.

*Phosphorylation of eIF2a*

When the α subunit of eIF2 (eIF2α) is phosphorylated at the serine-51, it becomes a competitive inhibitor of eIF2B through tight binding to the regulatory subunit of eIF2B.

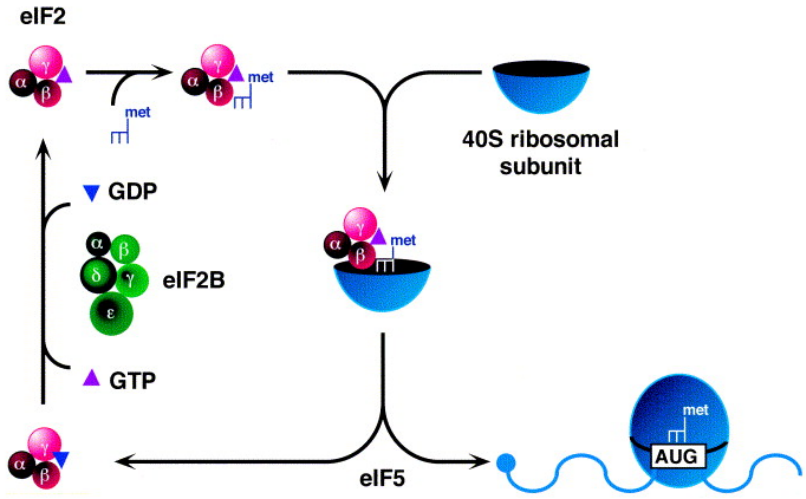


Figure 1.1: A scheme of eukaryotic translation initiation. Cited from Kimball, 1999.

This interaction inhibits the exchange of eIF2-GDP to eIF2-GTP and impairs the formation of TC, thus decreasing the rate of translation initiation and protein synthesis

(Krishnamoorthy et al., 2001). Therefore, phosphorylation of eIF2α is the critical control point for the rate of translation initiation. The phosphorylation site of eIF2α at serine-51 is conserved in eIF2α homologues from a wide range of eukaryotes. In animals, there are at least four different eIF2α kinases identified which are triggered through specific stimuli (Wek et al., 2006). In budding yeast, GCN2 is the only known eIF2α kinase. GCN2 plays an important role in cellular responses to amino acid availability. When amino acid availability is limited, there is an increase of the uncharged tRNA concentration. Uncharged tRNA interacts with GCN2 at its C terminal anticodon binding domain and causes autophosphorylation of GCN2 at threonine-882 and threonine-887 of the kinase domain, which in turn activates GCN2 kinase activity (Romano et al., 1998). Activated GCN2 phosphorylates eIF2α and causes a decrease in translation initiation rate, reducing the consumption of the limited amino acids. Although the general translation rate is decreased, there is an increase in the translation of GCN4 through a unique translation control mechanism (Hinnebusch, 1984). Therefore, GCN2 is activated during amino acid starvation in GCN2 kinase pathway. It phosphorylates its substrate eIF2α, which in turn decreases the global translation rate. Decreased translation rate induces the translation of the transcriptional activator GCN4, which activates the expression of amino acid biosynthesis genes (Figure 1.2).

## *Translational Control of GCN4*

### *Upstream Open Reading Frame (uORF)*

Sequence analysis of *GCN4* transcript reveals that the mRNA contains no introns and it has a 591 bp 5' UTR, in which there are four upstream open reading frames (uORFs;

Hinnebusch, 1984). These uORFs are only 2 to 3 codons in length and have their own start and stop codons. During translation initiation, the start codon of a uORF can be recognized by the scanning 40S ribosomal subunit and the associated TC and subsequently translated. There are three possibilities following the translation: 1) translation terminates at the stop codon of the uORF and re-initiates translation downstream, as is the case with the first uORF of the *GCN4* transcript; 2) translation terminates and the 40S ribosome dissociates with the mRNA, resulting in repression of translation of the downstream major open reading frame (mORF); or 3) synthesize an N-terminally extended protein(Meijer and Thomas, 2002). For the regulation of GCN4 translation, the first two possibilities occur.

*GCN4 is Regulated through the Presence of the uORFs*

Based on the scanning mechanism described above, each of the start codons of the four uORFs in the *GCN4* mRNA should be recognized by the scanning ribosomes during translation initiation prior to the start codon of the mORF. Studies have shown that removing the start codons of all four uORFs dramatically increase the translation of GCN4 mORF, indicating the function of uORFs as translational barriers (Mueller and Hinnebusch, 1986). In addition, misssense mutations of uORF1 have no phenotype, and when both uORF2 and uORF3 are removed, nearly wild-type phenotype is retained (Miller and Hinnebusch, 1989; Mueller and Hinnebusch, 1986). However, when the start codon of uORF1 is eliminated, the induction of GCN4 translation is impaired, indicating that uORF1 is a positive regulator. When there is only uORF3 or uORF4 alone present in the *GCN4* 5' UTR, GCN4 translation is strongly repressed (Hinnebusch, 2005). All these observations point to a complicated regulatory mechanism of GCN4 translation. As reviewed by Hinnebusch (to simplify the explanation, uORF2 and uORF3 are omitted in the model),

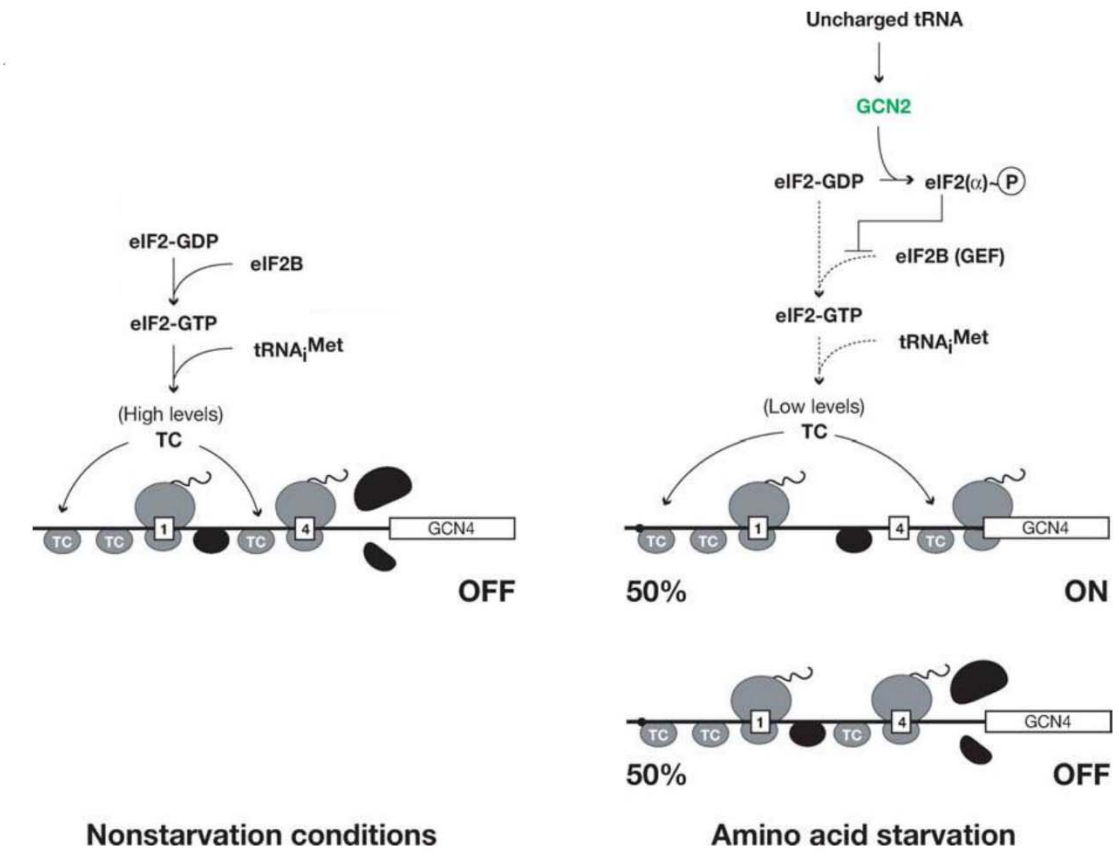


Figure 1.2: A model for GCN2 kinase pathway and GCN4 translational control. Modified from Hinnebusch 2005.

the 43S ribosomal subunit recognizes the start codon of uORF1, translates uORF1 and dissociates at the stop codon. The reinitiation of ribosomes downstream following translation of a uORF is usually inefficient (Hayden and Jorgensen, 2007; Kozak, 1984b). Therefore, only ~50% of the 40S ribosomes are still attached to the mRNA and continue scanning. In normal conditions (nonstarvation), when there is a high concentration of TC, the remaining 40S ribosomes quickly rebind to the TC, reinitiate translation at uORF4 and dissociate again after the translation of uORF4. Because of the distance between uORF4 and *GCN4* mORF, reinitiation does not occur at the start codon of *GCN4* mORF, thus

resulting in a low protein accumulation level of GCN4 (Abastado et al., 1991) (Figure 1.2). However, during amino acid starvation, phosphorylation of eIF2α significantly decreases the concentration of TC, lowering the rate of the rescanning 40S ribosomes to rebind TC. As a result, approximately 50% of the rescanning 40S ribosomes bind to TC when they scan past uORF4 due to the lengthened time it takes to rebind TC, and reinitiate at the start codon of *GCN4* mORF instead. Thus, amino acid starvation triggers GCN2 to phosphorylate eIF2α, which in turn allows for ribosomes to overcome the inhibitory effects of uORFs in the *GCN4* mRNA leader and results in a dramatic increase of GCN4 protein accumulation (Hinnebusch, 1997, 2005).

*The Similarities between GCN4 and O2*

There are structural and functional similarities between GCN4 and O2. Structurally, the *O2* transcript has a 258 bp 5' UTR that contains 3 uORFs compared to the *GCN4* transcript which has 4 uORFs (Figure 1.3) (Lohmer et al., 1993; Schmidt et al., 1990). uORF2 overlaps with uORF3 by 20 bp in *O2*, which is different from *GCN4*. Similar to GCN4, the uORFs of the *O2* transcript are also described to negatively regulate the translation of O2, as mutations that disrupt the start codons of one or more uORFs significantly increase the O2 translation *in vivo* using an engineered reporter system in tobacco (Lohmer et al., 1993). Functionally, as described above, both GCN4 and O2 are b-zip transcription factors that activate the expression of many target genes when induced. Furthermore, O2 can partially rescue the yeast *gcn4* mutant grown in the presence of a *HIS3* gene product inhibitor, indicating that the *HIS3* gene is activated by O2 through its GCN4 binding site (Mauri et al., 1993). Despite the similarities described above, there has been no evidence demonstrating O2 as a GCN4 homologue in maize and as part of the

GAAC response. In fact, no homologues of GCN4 have been identified in plants so far (Halford, 2006). In mammalian cells, it has been shown that the translation of b-zip transcription factor activating transcription factor 4 (ATF4) is induced by phosphorylation of eIF2α during amino acid starvation and ER stress, triggering increased expressions of genes involved in multiple stress response pathways (Harding et al., 2000a; Kaufman et al., 2002). The enhanced translation of ATF4 is by overcoming the inhibitory effect of the second uORF whereas the first uORF serves as a positive regulator (Vattem and Wek, 2004). These studies suggest that O2 is likely a GCN4 homologue in maize and its translation is induced by phosphorylation of eIF2α in amino acid starvation as part of the GAAC response.

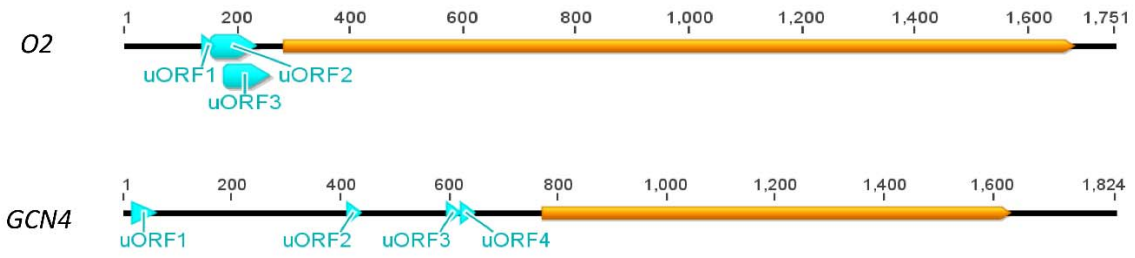


Figure 1.3: Schematic view of maize O2 (upper) and yeast GCN4 (lower) uORF structures.

*GCN2 Kinase Pathway in Other Organisms*

GCN2-like proteins are well conserved among eukaryotes. In animals, GCN2 homologues have been described in *Drosophila melanogaster* and mammalian systems such as mice, rats and human cells (Berlanga et al., 1999; Chotechuang et al., 2009; Harding et al., 2000a; Santoyo et al., 1997). In *Drosophila*, DGCN2 expression is regulated developmentally. DGCN2 is expressed in several tissues during embryogenesis and is

restricted to several cells in the central nervous system in the later developmental stages. DGCN2 is shown to phosphorylate an eIF2α substrate *in vitro*. However, it is not clear whether the kinase activity of DGCN2 can be activated during amino acid starvation (Santoyo et al., 1997). In mammalian systems, *gcn2* knockout mutant mice grow normally and exhibit comparable phenotypes as wild type mice in standard growing conditions; whereas during amino acid deprivation, mortality rate of *gcn2* mutant mice significantly increases (Zhang et al., 2002). Berlanga et al. reported that GCN2 phosphorylates eIF2α during serum starvation in mouse GCN2 transfected human cells (Berlanga et al., 1999). Harding et al. reported that GCN2 is activated by amino acid starvation and phosphorylates eIF2α in mouse embryonic cells, which causes an induction of ATF4 translation and activation of genes involved in amino acid biosynthesis (Harding et al., 2000a). These studies suggest that the GCN2 kinase pathway and GAAC response are conserved in mammals.

In plants, GCN2 homologues have been reported in *Arabidopsis* and wheat (Byrne et al., 2012; Lageix et al., 2008; Li et al., 2013; Zhang et al., 2003; Zhang et al., 2008a). GCN2 was first cloned in *Arabidopsis*, reported to have conserved protein functional domains as its homologues in yeast and animals (Zhang et al., 2003). It shares similar biochemical properties to the yeast and animal homologues and is shown to interact with uncharged tRNA and phosphorylate eIF2α *in vitro* (Li et al., 2013). *Arabidopsis gcn2* knockout mutant seedlings grow normally in standard conditions but exhibit more sensitivity when treated with amino acid biosynthesis inhibitors (Zhang et al., 2008a). AtGCN2 phosphorylates eIF2α during amino acid starvation but whether it affects the expression of amino acid biosynthesis genes is unknown. In addition to amino acid

starvation, AtGCN2 was activated by a variety of stress stimuli, such as purine starvation, UV radiation, cold shock, wounding, and methyl jasmonate and 1-aminocyclopropane-1-carboxylic acid treatment, which leads to a reduction in global protein synthesis (Lageix et al., 2008). The activation of AtGCN2 by wounding and the plant defense related compound and hormone suggests that AtGCN2 could function in plant defense against insects (Lageix et al., 2008). However, AtGCN2 could not be activated by viral infections with *Turnip yellow mosaic virus* or *Turnip crinkle virus*, neither is eIF2α phosphorylated in these cases, suggesting that phosphorylation of eIF2α may not play a role in plant response to viral infection, unlike the animal eIF2α homologues (Chou et al., 1995; Williams, 1999; Zhang et al., 2008a). In wheat (*Triticum aestivum*), overexpression of TaGCN2 significantly reduces the total free amino acid concentration, with free asparagine concentration being substantially reduced in particular. TaGCN2 is also reported to regulate genes such as encoding aspartate kinase / homoserine dehydrogenase and 3-deoxy-D-arabino-heptulosonate-7-phosphate synthase in response to sulfur deprivation, which has not been shown in other plants nor fungi (Byrne et al., 2012). Together, these observations suggest that GAAC system is at least partially conserved in plants, but compared to animals and fungi, GCN2 is not only activated by amino acid starvation but also by other stimuli and stresses.

### *Overview*

In this work I focus on characterization of maize GCN2 kinase and GAAC in maize, GCN2 regulation of O2 and the role of GCN2 in maize growth and development in response to abiotic stresses. In chapter one I present a study that combines transcription profiling and proteomics to investigate the pleiotropic effects of *o2* mutation, which

demonstrates that the increase of certain lysine-rich protein accumulation, such as glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and sorbitol dehydrogenase (SDH1), could contribute to the overall elevated lysine content in *o2* kernels. Genes that are also affected by *o2* mutation include amino acid biosynthesis genes, plant defense genes and starch biosynthesis genes. Chapter two focuses on characterization of maize GCN2 and its regulation of O2 during amino acid starvation. I show that eIF2α is only phosphorylated in the wild type maize but not in *gcn2* mutants when amino acid starvation is induced, and that the up regulation of O2 protein accumulation is associated with increased eIF2α phosphorylation. These observations indicate that GCN2 kinase pathway and GAAC are at least partially conserved and that O2 could be a GCN4 homologue in maize. In chapter three I investigate the role of GCN2 in response to other abiotic stresses, such as drought. Surprisingly, the *gcn2* mutant seedling is more tolerant to polyethylene glycol induced drought stress compared to wild type. RNA-Seq reveals that several ABA inducible genes are up regulated in *gcn2* mutant leaves. I also show that the endogenous level of abscisic acid (ABA) is elevated in *gcn2* mutant leaves compare to wild type and that drought treatment does not induce a significant increase in *gcn2* mutant leaves. Chapter four studies the possible role of eukaryotic initiation factor 5A (IF5A) in 27 kDa γ-zein mRNA translation and retention in the ER lumen, where I demonstrate that IF5A could play a role in 27 kDa γ-zein mRNA localization by binding to its 3' UTR and associating with an actin-rich cytoskeletal fraction in maize endosperm.

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CHAPTER TWO

# Identification and Characterization of Lysine-Rich Proteins and Starch Biosynthesis Genes in the opaque2 Mutant by Transcriptional and Proteomic Analysis*

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*Abstract*

The *opaque2* mutant is valuable for producing maize varieties with enhanced nutritional value. However, the exact mechanisms by which it improves protein quality and creates a soft endosperm texture are unclear. Given the importance of improving nutritional quality in grain crops, a better understanding of the physiological basis for these traits is necessary. In this study, we combined transcript profiling and proteomic analysis to better understand which genes and proteins are altered by *opaque2* in the W64A inbred line. These analyses showed that the accumulation of some lysine-rich proteins, such as sorbitol dehydrogenase and glyceraldehyde3-phosphate dehydrogenase, was increased in mature kernels and may contribute substantially to the lysine content of *opaque2* endosperm. Some defense proteins such as beta-glucosidase aggregating factor were strongly down regulated and may be regulated directly by *opaque2*. The mutant also had altered expression of a number of starch biosynthesis genes and this was associated with a more highly crystalline starch. The results of these studies revealed specific target genes that can be investigated to further improve nutritional quality and agronomic performance of high lysine maize

lines, particularly those based on the presence of the *opaque2* mutation. Alteration of amylopectin branching patterns in *opaque2* starch could contribute to generation of the soft, starchy endosperm.

## *Introduction*

Maize is a major food and feed crop, and the acreage devoted to maize cultivation is expected to increase significantly over the next several decades due to greater demand for the grain (Pingali, 2001). The majority of the maize crop is used to feed livestock, but in substantial parts of Central America, Africa and Asia, maize is the primary food staple for humans. In order to maximize land productivity, the nutritional quality of crops should be one of the factors considered, along with water and nitrogen use efficiency, yield, pest resistance and other determinants of crop productivity (Tester and Langridge, 2010).

Maize protein is deficient in the essential amino acids lysine and tryptophan, which limits its value for monogastric animals. Therefore, for the past several decades there have been efforts to create maize lines with increased essential amino acid content. In the 1960s the research groups of Mertz and Nelson at Purdue University identified several mutants with increased lysine content, *opaque2* (*o2*) and *floury2* in particular had substantially higher essential amino acid content (Mertz et al., 1964; Nelson et al., 1965). However, these mutations result in a soft, chalky endosperm phenotype that is not suitable for agronomic production because of increased susceptibility to insect and fungal pests and decreased yields (Schoonho.Av et al., 1972; Warren, 1978). The *O2* gene was found to encode a b-zip transcription factor (Schmidt et al., 1990) that regulates expression of several genes in the endosperm, notably those encoding the 22 kDa α-zein storage proteins (Schmidt et al., 1992). The substantial reduction in synthesis of α-zeins results in smaller,

less numerous protein bodies and a concomitant increase in non-zein endosperm proteins (Mertz et al., 1964). These changes in protein accumulation result in an endosperm that has nearly twice the lysine and tryptophan content of wild-type maize (Mertz et al., 1964), which substantially improves its value for monogastric animals (Mertz et al., 1965). Therefore, breeders began recurrent selection of *o2* lines with high lysine and a hard endosperm, called Quality Protein Maize (Vasal et al., 1980).

Recently, considerable progress has been made developing maize lines and optimizing amino acid balance using transgenic (Huang et al., 2006; Huang et al., 2005; Segal et al., 2003) and conventional breeding approaches through marker-assisted selection (Scrimshaw, 2006; Vivek et al., 2008). The most successful transgenic strategies have been specific knock down of zein storage protein or lysine catabolism gene expression with RNA interference (RNAi) approaches (Hournard et al., 2007; Huang et al., 2006; Huang et al., 2005; Segal et al., 2003). Reduced synthesis of the lysine-poor zein proteins and compensatory increases in other proteins dramatically improves the nutritional quality of the grain. The underlying mechanism for rebalancing amino acid content for both *o2* and RNAi is unclear, although it depends on reduced synthesis of the zein storage proteins and a compensatory increase in non-zein protein content (Wu and Messing, 2012). Generally, the total protein content is only slightly depressed relative to wild type kernels (Gutierrez-Rojas et al., 2008; Huang et al., 2005; Wu and Messing, 2012), and knocking down 19- and 22-kDa α-zeins in high or low protein lines by RNAi only modestly changes total protein content from the parental levels, suggesting that total protein content is under genetic control (Wu and Messing, 2012). It is possible that competition between mRNA

transcripts for ribosomes is responsible for the final protein composition, as has been proposed for soybean (Schmidt and Herman, 2008).

Despite these advances in developing maize lines with higher nutritional value, the underlying physiological and molecular mechanisms that cause soft kernels is still not well understood. Several studies have investigated the changes in transcriptional patterns caused by the *o2* mutation (Frizzi et al., 2010; Hartings et al., 2011; Hunter et al., 2002; Jia et al., 2007). Consistent observations among them point to pleotropic changes in gene expression, but it has been difficult to identify physiological pathways that explain the soft kernel phenotype and changes in protein synthesis that contribute to the improved amino acid composition of the endosperm. Genes related to endoplasmic reticulum (ER) stress responses are consistently up regulated in opaque mutants (Hunter et al., 2002), as are many genes in the glycolytic pathway and others that are typically associated with physiological responses to anoxic stress, such as alcohol dehydrogenase and sorbitol dehydrogenase (Frizzi et al., 2008; Hartings et al., 2011; Hunter et al., 2002), but their roles in the expression of the opaque phenotype are not clear. Proteomic analysis of protein accumulation during *o2* development is generally consistent with the pattern of gene expression observed by microarray analysis (Damerval and Le Guilloux, 1998).

In this study we analyzed gene expression in *o2* endosperm using an amplified fragment length polymorphism (AFLP)-based approach that is open-ended and does not depend on known or predicted gene sequences. We also performed a proteomic analysis of mature seeds to identify specific proteins that contribute disproportionately to the increased lysine and tryptophan content in order to relate these more abundant gene products to gene expression in maturing endosperm. In addition to confirming overall gene expression

patterns previously described for *o2* mutants, we identified a number of other differences in mRNA transcript levels compared to wild type endosperms. Several gene products related to defense responses were also substantially down regulated in *o2* endosperm, which could further explain its greater susceptibility to ear rots and insect pests. Expression of starch biosynthetic genes was altered in *o2* and it could be associated with changes in starch granule structure. Furthermore, analysis of protein accumulation in mature seeds revealed a few lysine-rich proteins that were substantially more abundant in *o2* endosperm. These changes could explain a significant fraction of the increased lysine content in W64A*o2*. How changes in gene expression, protein content and starch structure contribute to the development of opaque endosperm is discussed.

## *Materials and Methods*

### *Transcript Profiling by GeneCalling™*

Plants of the nearly-isogenic maize (*Zea mays L.*) inbred lines W64A + and W64A*o2* (Hunter et al., 2002) were grown in the summer of 1998 in field plots at the Pioneer Hi-Bred International genetic nursery in Johnston, IA. Well-filled ears of each inbred line were harvested 22 days after pollination (DAP) and immediately frozen in liquid nitrogen. To minimize the effect of biological variation between ears on the gene expression analysis, equal numbers of endosperms from the middle portion of three ears were pooled. Total RNA was isolated using the PUREscript kit (Gentra Systems, Inc., Minneapolis), and mRNA profiling was performed at Curagen (New Haven, CT) by GeneCalling™ (Shimkets et al., 1999). In brief, cDNA was synthesized from three independently pooled W64A + and three independently pooled W64A*o2* endosperm

samples (biological repetitions). Each of the six cDNA preparations was divided into three aliquots (technical repetitions) to provide nine repetitions per genotype for profiling analysis. Each cDNA aliquot was digested with 47 different combinations of restriction enzyme pairs. Fragments from each digest were ligated to adapters; the fragments were amplified with primers that have unique tags (biotin on one end, fluorescent marker at the other). Labeled fragments were purified using streptavidin beads and resolved by high-resolution gel electrophoresis to generate traces showing peaks whose position and height represented $M_r$ and abundance of cDNA fragment(s), respectively. GeneCalling™ software compiled a list of differentially abundant fragments and assigned a ranking (significance) to each detected difference. The software further searched a nucleic acid database for fragments with the same length and end sequences and predicted likely gene candidates. The identity of predicted fragments was confirmed by competitive amplification with an unlabeled gene-specific primer ("poisoning") or by cloning and sequencing the fragment (Shimkets et al., 1999). The confirmed sequences are provided in the supplemental section of this chapter (Supplemental File 1).

*Confirmation of Expression Differences by Quantitative Real-Time PCR*

*Plant materials.* W64A + and o2 kernels for quantitative polymerase chain reaction (qRT-PCR) and western blotting were grown in Elm Mott, TX during the summer of 2012. The kernels were harvested at 22 DAP and kept frozen at −80 °C. Three ears of each genotype were used as three biological replicates. Six endosperms of each ear were dissected and ground to a fine powder in liquid nitrogen using a mortar and pestle. For

RNA isolation, up to 0.1 g of the materials were used. For protein extraction, 50 mg were weighed and homogenized in borate extraction buffer (12.5 mM NaBO3, 1% (w/v) sodium dodecyl sulfate and 2% (v/v) 2-mercaptoethanol).

*RNA isolation, cDNA synthesis and qRT-PCR.* Total RNA was isolated from frozen kernels using Purelink™ Plant RNA Reagent (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. RNA samples were diluted to a final concentration of ~100 ng/µl and quantified on a NanoDrop ND-1000 UV/Vis spectrophotometer (NanoDrop Technologies, Wilmington, DE), the purity of which was checked by the ratio of absorptions at 260 nm and 280 nm and all the samples had a ratio ≥ 2.0. First-strand cDNA was synthesized from 1 µg of RNA using qScript™ cDNA SuperMix (Quanta Biosciences, Gaithersburg, MD) and subsequently diluted 10-fold in water.

Primers for qRT-PCR were designed to amplify a 150–300 bp region of selected genes based on Primer3 Plus software (www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi). Primers were designed for a 62 °C annealing temperature and to span exon-exon junctions in order to control for genomic DNA contamination. The list of primer sequences is provided in supplemental Table S2.1.

For gene expression analysis, qRT-PCR was performed in a 72-well rotor using the Corbett Rotor-Gene™ 3000 (Qiagen, Velancia, CA). Each 20 µl reaction contained 10 µl PerfeCTa® SYBR® Green FastMix® (Quanta Biosciences, Gaithersburg, MD), 2.5 µl 10-fold diluted cDNA or 1 µl plasmid standards with copy numbers from 105 to 108, and 1 µM of each primer. The PCR program was as follows: 50 °C hold for 2 min for auto gain optimization, 95 °C initial denaturing for 10 min, 50 cycles of 95 °C for 15 s and 60 °C for 1 min. Melting curves were obtained by heating from 55 °C to 95 °C with a 1 °C per second

ramp rate to confirm single amplicons. Expression levels of genes in W64A + and W64Ao2 were normalized against the expression of RRB1 gene in the corresponding genotypes (Sabelli et al., 2005), since it was not differentially expressed between the two genotypes in preliminary experiments. Normalization of Gene expression was performed using the Q-Gene Core Module file (Simon, 2003). Statistical differences of gene expression levels between W64A + and W64A*o2* were evaluated with unpaired two-tailed student's t-test, and the agreement of gene expression levels from transcript profiling and qRT-PCR results were calculated with Pearson correlation coefficient with significance determined by ANOVA, using the JMP statistical software (SAS Institute Inc., Cary, NC).

*Kernel Protein Extraction, SDS-PAGE and Western Blotting*

Total soluble proteins from maize kernels were extracted with borate extraction buffer containing 12.5 mM sodium borate, 1% (w/v) SDS, 2% β-mercaptoethanol, pH 10 (Wallace et al., 1990). One ml of borate extraction buffer was added to 50 mg ground kernels and incubated with shaking for at least 2 h at room temperature. Insoluble cell debris was removed from the crude extract by centrifugation for 15 min at 16,000 × g at room temperature. The cleared protein extracts were aliquoted and stored at −80 °C.

Twenty-five μg of total protein from each sample were separated by 12% SDS-PAGE in 1X SDS-PAGE running buffer (25 mM Tris, 192 mM glycine, 0.1% (w/v) SDS) and then transferred to a BioTrace™ PVDF membrane (Pall Corporation, Pensacola, FL) using a TE 22 Mighty Small Transphor Tank Transfer Unit (GE Healthcare, Piscataway, NJ). The quality of protein transfer was visually checked using pre-stained protein markers (Precision Plus Protein™ All Blue standards, Bio-Rad, Hercules, CA) and staining the membrane with Ponceau S (0.1% (w/v) in 5% (v/v) acetic acid). The membrane was

blocked with 3% non-fat dry milk powder in 1X TBST buffer (10 mM Tris–HCl, pH 8.0, 150 mM NaCl, 0.1% (v/v) Tween-20) for 1 h at room temperature with shaking.

Primary antibodies for immunoblots were as follows: RPS6 provided by Julia Bailey-Serres; BGAF provided by Asim Esen; GAPDH provided by Ming-Che Shih; eIF2α, eIF4E, and eIF4G provided by Karen Browning; SSIIa provided by Hanping Guan; SSIII, BEI and BEIIa/b provided by Alan Myers; anti-actin mouse monoclonal antibody (Cat. No. A0480, Sigma St. Lois MO). Membranes were incubated with primary antibodies diluted in TBST (1:1000 to 1:3000, based on the antibody titer) for 1 h at room temperature or overnight at 4 °C, washed with TBST and then incubated for 1 h at room temperature with secondary antibodies (horseradish peroxidase-conjugated goat anti-rabbit or goat anti-mouse; Invitrogen, Carlsbad, CA) diluted in TBST (1:30,000). After washing with TBST, the membrane was treated with 1 ml SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL) for 2 min and the signals were detected using the Ultra- LUM Gel Imager System and UltraQuant 6.0 software (Ultra-Lum, Incorporated, Claremont, CA). The intensity of bands was quantified using the ImageJ software (Schneider et al., 2012) and statistical differences of protein expression levels between W64A + and W64A*o2* were evaluated with unpaired two-tailed student's t-test with the JMP statistical software (SAS Institute Inc., Cary, NC).

*2D SDS-PAGE*

Endosperms from mature kernels were isolated by soaking overnight in ddH2O at 4°C. Pericarp and embryo were removed and the endosperms dried in a freeze dryer; dried endosperms were ground to flour with a bead mill. Flour samples were extracted in borate extraction buffer with shaking overnight at 37°C (Wallace et al., 1990). Protein extracts

were fractionated into zein and non-zein fractions by precipitation in 70% ethanol; the non-zein protein pellet was washed twice with 70% ethanol, dried and resuspended in IPG rehydration buffer (8 M urea, 2% CHAPS, 20 mM dithiothreitol, 0.005% bromophenol blue). Samples were loaded into immobilized pH 4–7 gradient strips directly during the rehydration of the gel. The first dimension separation was performed according to the manufacturer's directions on either a Multiphor II or Ettan IPGphor 2 (GE Healthcare, Piscataway, NJ). The second dimension separation was performed using the Mini-Protein II vertical gel apparatus (Bio-Rad, Hercules, CA) according to the manufacturer's directions. Proteins were visualized with Coomassie brilliant blue. Gels were compared and spot intensities quantified using Prodigy SameSpots gel analysis software (Nonlinear Dynamics, Newcastle upon Tyne, UK).

*Protein Identification*

Protein spots of interest were excised from the acrylamide gel and digested with trypsin using an in-gel digestion procedure. Briefly, gel pieces were destained by incubating in 50% acetonitrile 15 min at room temperature with two to three changes of solution. The gel pieces were dried in a Speed-Vac drier for 1 h, and were rehydrated by incubating in trypsin digestion buffer (50 mM ammonium bicarbonate, 5 mM $CaCl_2$, 15 µg/ml sequencing grade trypsin (Sigma, St. Louis, MO)) for 10 minutes. Excess buffer was removed and enough trypsin-free digestion buffer was added to barely cover the gel pieces; the samples were then incubated overnight at 37°C. The buffer solution was removed to a fresh tube and the gel pieces were washed two times by incubating in 50% acetonitrile with 1% formic acid for 5 minutes and combined with the original supernatant. Peptides were

concentrated and de-salted using C-18 Zip-Tips (Milipore, Billerica, MA) according to the manufacturer's instructions.

Protein identification was performed by MALDI-TOF peptide mass mapping at the University of Arizona Mass Spectrometry facility. The peak lists derived from the mass spectra were searched against the Genbank non-redundant database updated 7/1/11 using the ProFound peptide mapping tool ((Zhang and Chait, 2000); http://prowl.rockefeller.edu/). Searches were performed using monoisotopic masses with the following parameters: taxonomy, other green plants; constant modification, iodoacetamide; Partial modification, methionine oxidation; mass tolerance, 200 ppm.

*Analysis of Starch Structure*

Starch granules were purified essentially as described by Gutierrez et al. (Gutierrez et al., 2002). Mature kernels were soaked in 0.5% $Na_2S_2O_5$ at 50°C for 24 h. The endosperm was dissected from the pericarp and germ, and ground lightly in a mortar. The sample was blended with 50 mM NaCl for 30 s and filtered through two layers of Miracloth (Calbiochem, San Diego, CA). The filtered material was extracted and pelleted by centrifugation five times in 1:4 toluene: 50 mM NaCl, followed by extraction two times with acetone. The starch was dried for 48 h before use.

Dried starch was weighed and suspended in a 1:3 [w:v] slurry in deionized water. The slurry was sealed in hermetic pans for DSC. The sample pan was loaded into the DSC instrument (Q200, TA Instruments, New Castle, DE) sample pedestal and the reference pedestal held an empty hermetic pan. The sample was equilibrated at 35 °C and then a DSC scan was performed from 35–95 °C heating at 5 °C/min. The onset temperature, peak

endotherm and total enthalpy were calculated using the TA Universal Analysis 2000 software (TA Instruments, New Castle, DE).

Amylopectin glucan chain length distributions were determined by capillary electrophoresis. Starch was dissolved in DMSO by heating to 95°C for 15 minutes. A small sample of the dissolved starch was debranched by isoamylase (Megazyme, Wicklow, Ireland) and the resulting glucans were labeled with 8-amino-1,3,6-pyrenetrisulfonic acid in the presence of sodium cyanoborohidride. A sample of the labeling reaction was diluted and the fluroescently labeled glucan chains were separated and quantified by fluorescence-assisted capillary electrophoresis. Histograms of the percentage of area for each peak were plotted and compared.

## *Results*

### *Overview of Transcript Profiling*

To systematically compare gene expression patterns between W64A + and W64A*o2* at the most metabolically active stage of endosperm development, transcript profiling was performed at Curagen Corp. (New Haven, CT) by GeneCalling™ (Shimkets et al., 1999) at 22 DAP. The GeneCalling™ approach does not rely on a priori knowledge of gene sequences and can therefore identify expression differences for genes that are not present in sequence databases. cDNA fragments were generated with 47 different pairs of restriction enzymes, and the expression levels of the corresponding gene fragments were compared. A total of 470 putative genes were identified as differentially expressed in W64A + and W64A*o2* by the GeneCalling™ software using a t-test. The sequence of a subset of the differentially expressed gene fragments was confirmed by oligonucleotide

competition, “poisoning”, with an unlabeled gene-specific primer or by cloning and sequencing the fragments if poisoning failed. The identities of 274 gene fragments ranging from 50 to 500 bp were confirmed and represented a total of 151 gene products. Further characterization of these genes was obtained by BLASTN and BLASTX analyses against Genbank and Maize Genome Sequence databases (http://www.maizesequence.org). The molecular functions and biological processes were annotated using the gene ontology database (G.O.; http://www.geneontology.org) and classification of their molecular functions and biological processes are illustrated in Figure 2.1. A comprehensive table of differentially expressed genes and their properties is provided in supplemental Table S2.2. Twenty-six distinct biological functions were affected in W64Ao2, including carbohydrate metabolism and stress responses, which are associated with the altered endosperm phenotype of the *o2* mutant. Specifically, 70 genes corresponding to 23 functional groups were up regulated and 81 gene fragments belonging to 16 groups were down regulated in *o2*.

*GO Functional Classes of Up Regulated and Down Regulated Genes*

As expected, a large number of down regulated genes have a molecular function associated with nutrient reservoir activity (Figure 2.1), which is due to the accumulation of several 19- and 22-kD α-zein genes and the 27-kD γ-zein being significantly reduced in *o2*. These proteins are encoded by large gene families with highly conserved sequences and are well-characterized targets of O2 regulation (Song and Messing, 2002). Reduction in nutrient reservoir gene function in *o2* is followed by catalytic activity (23%), structural molecule activity (8%), protein binding (8%), DNA binding (6%), transporter activity

(4%) and other minor categories (4%), such as ion binding and enzyme regulator activity. Among biological processes, 34% of the down regulated genes participate in the secretory pathway, which may reflect a response to the reduced

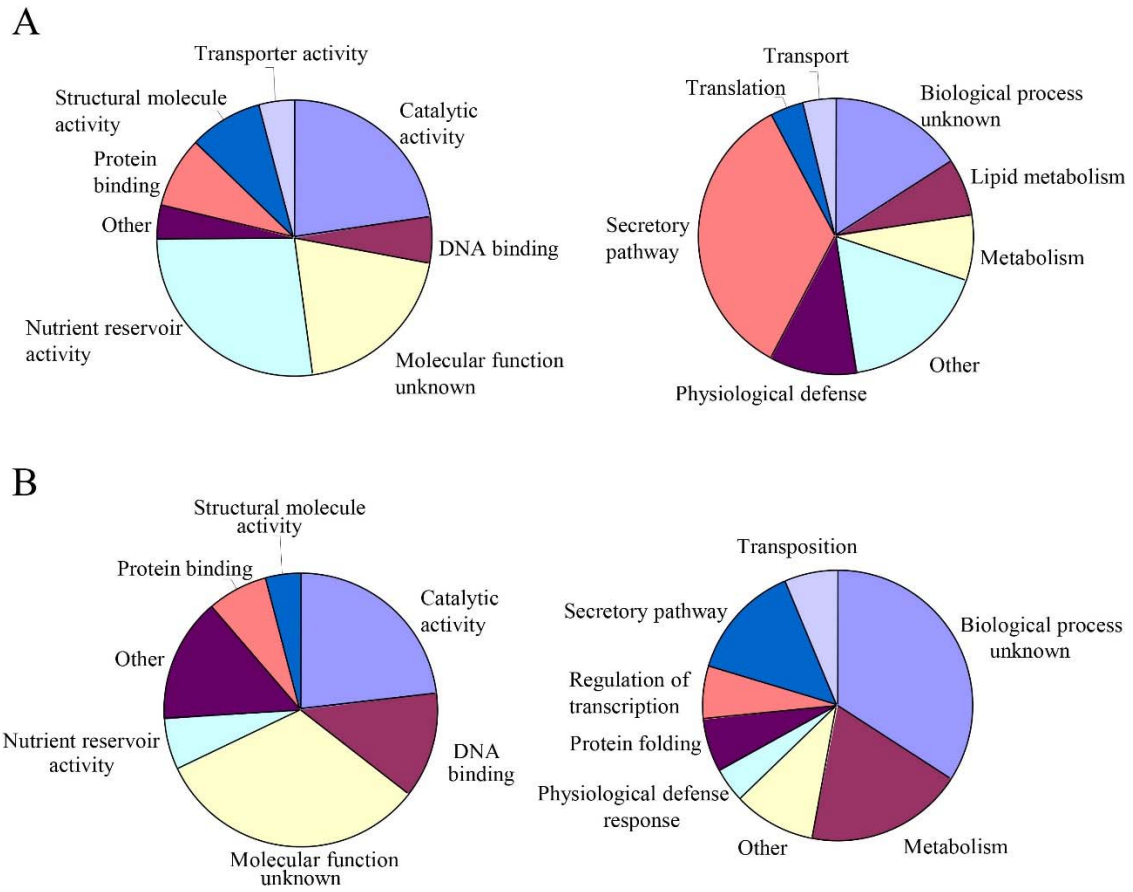


Figure 2.1: GO classification for genes with altered expression in *o2*. Genes were assigned to GO molecular function (A) and biological process (B). The plots on the left are genes down regulated in *o2* and the plots on the right are genes up regulated in *o2*.

accumulation of the ER-resident zein storage proteins. Other down regulated functional categories include physiological defense (11%), metabolism (8%), lipid metabolism (7%), transport (4%), translation (4%) and other (17%). There are 20% and 16% of the genes with unknown molecular function and biological process, respectively.

For genes that are up regulated in *o2*, only a small proportion have the function of nutrient reservoir activity (6%). Instead, the largest proportion of the up regulated genes have catalytic activities (23%), followed by DNA binding (13%), protein binding (7%), structural molecule activity (4%) and other (15%). This is consistent with metabolism being the most affected biological function (19%), followed by secretion (14%), protein folding (6%), transcription (6%), transposition (6%), and physiological defense response (4%). Of the up regulated genes, 32% could not be assigned a molecular function and 34% could not be assigned to a biological process.

*Genes Down Regulated in o2*

As expected, members of the zein gene family were significantly down regulated in *o2* (19 kDa and 22 kDa α-zeins and 27 kDa γ-zein). There were a few zein genes with increased expression in o2. However, this method of analyzing transcripts is very sensitive to allelic differences, and the up regulated zein genes may represent such alleles. Several genes that are reported to participate in defense responses to biotic and abiotic stresses were also significantly down regulated in *o2*, such as a ribosome-inactivating protein (RIP) b-32, which has a defensive role against pathogens and viruses and a well-known target of O2 regulation in maize (Bass et al., 1992; Hey et al., 1995). A beta-glucosidase aggregating factor-like protein (BGAF) was also strongly down regulated; such proteins are reported to be involved in defense against pathogens and herbivores (Blanchard et al., 2001; Kittur et al., 2007). The BGAF-like protein may be a particularly interesting gene to study further because it has an O2 consensus binding sequence (Frizzi et al., 2008; Yunes et al., 1998) at −227 nt from the predicted transcription start. However, there were several other defense-related transcripts that were down regulated to a lesser extent, subtilisin-

chymotrypsin inhibitor CI-1B (CI-1B), which responds to wounding (Habib, 2007), flower-specific gamma-thionin (defensin SD2), which is toxic to animal cells and defends against parasites (Ganz, 2003), and basal layer antifungal protein2 (BAP2). It is possible that the high sensitivity of *o2* to fungal and insect pests is due to the synergistic effect of reducing both b-32 and BGAF protein levels in *o2* endosperm.

Several ribosomal proteins, such as the 40S subunit protein S3a and the 60S ribosomal subunit protein L19-3, and the 18S RNA gene, the structural RNA for the small subunit of eukaryotic cytoplasmic ribosomes, were all down regulated in *o2*. Some of these changes in ribosomal constituents may be cellular responses to the changes in the overall mRNA pool, which lacks the abundant ER-targeted α-zein mRNAs in *o2*. Other down regulated transcripts included NAC (NAM, ATAF, and CUC transcription factor) domain-containing protein 48, which is predicted to function as a plant specific transcription factor involved in a variety of developmental events, as well as in biotic and abiotic stress responses (Olsen et al., 2005). Genes that function in signal transduction, such as YT521-B-like family protein, glutathione S-transferase GST 31, protein FAR-RED IMPAIRED RESPONSE 1, also showed decreased expression in *o2*. The role of these transcription factors and signal transduction proteins have in the formation of the opaque phenotype, if any, is unclear.

Several genes that function in amino acid metabolism were also down regulated, including tryptophan aminotransferase (TA1) and ketol-acid reductoisomerase which catalyzes two steps of the biosynthetic pathway of the branched-chain amino acids valine, leucine and isoleucine (Durner et al., 1993) and alanine-glyoxylate aminotransferase 2. Surprisingly, LKR-SDH1 is thought to be regulated by O2 (Kemper et al., 1999), yet the

transcript expression was not significantly different between W64A + and W64A*o2* and this may indicate that its expression is influenced by genetic background or environment. It may be the case that a large number of amino acid biosynthetic enzymes are regulated to some extent by O2. The yeast homolog of O2, the b-zip transcription factor GCN4 (General Control Non-derepressible 4), is known to induce the expression of a large number of amino acid biosynthetic genes in response to amino acid starvation (Hinnebusch, 1986) and gcn4 mutants can be complemented by expression of the maize O2 gene (Mauri et al., 1993).

Several genes related to cell structure and development were down regulated in W64A*o2*, including: katanin p60 ATPase which is involved in the regulation of microtubule dynamics (Nakamura et al., 2010) and regulates plant cell division and growth (Panteris et al., 2011); arabinogalactan protein (AGP), which serves as a marker of cellular identity and fate, and functions in plant vegetative growth and development as well as secondary cell wall thickening and programmed cell death (Showalter, 2001); brassinosteroid biosynthesis-like proteins, which are natural growth regulators required for post-embryonic growth (Clouse and Sasse, 1998);and maternal effect embryo arrest 21 (MEE21), which regulates embryo development and maturation (Pagnussat et al., 2005). Although it is not known how such proteins influence the opaque phenotype, it is possible that they could cause changes in cellular organization that predispose the endosperm cells to develop the characteristic gaps between starch granules that is a hallmark of opaque endosperm.

*Genes Up Regulated in o2*

A number of genes encoding primary carbohydrate metabolism enzymes were up regulated in W64A*o2*. Two enzymes of the glycolytic pathway were up regulated, cytosolic triosephosphate isomerase (TIM) and cytosolic phosphoglycerate kinase (PGK). Fructokinase-1, which functions at the entry point into glycolysis via the formation of glucose-6-phosphate and maintains the flux of carbon towards starch formation, was increased 1.85-fold. Many of these changes in glycolytic enzyme expression and the up regulation of alcohol dehydrogenase 1 by 2.86-fold were consistent with a hypoxic response. It has been shown that the maize endosperm is a highly anoxic environment compared to the embryo, and that this is likely to result in the shunting of carbon into starch rather than oil (Rolletschek et al., 2005). However, it is not clear why the *o2* mutant would display increased hypoxic responses, and the proportion of starch in *o2* endosperm is essentially identical to wild type in the W64A background (not shown).

Enzymes involved in starch biosynthesis were increased in *o2*, including granule-bound starch synthase I (GBSSI), which is required for the synthesis of amylose. Enzymes required for amylopectin synthesis were also up regulated, including pullulanase-type starch debranching enzyme1 (Zpu1), which hydrolyzes the α-1,6-glucosic linkages of polyglucans, 1,4-alpha-glucan-branching enzyme 2 (BE2), which catalyzes the formation of α-1,6 glucan and is required for amylopectin synthesis at the surface of the starch granule. Trehalose-6-phosphate synthase was also increased, which has been implicated in the redox activation of ADP-Glc phosphorylase, the enzyme that catalyzes the first committed step of starch synthesis (Eastmond et al., 2002). Prior work has shown that the biochemical properties of starch are altered in opaque mutants (Gibbon et al., 2003), but

the underlying mechanism is still not clear. The change in expression of one or more starch biosynthesis enzymes could result in the observed properties of *o2* starch, although altering the expression or mutation of one starch biosynthetic enzyme can have complex effects on multiple enzyme activities.

Several proteins involved in the maintenance and folding of proteins in the ER were up regulated. The expression of the calcium-dependent protein chaperones, Calnexin, calreticulin2 and the chaperone DNA J2, were increased approximately two-fold in W64A*o2*. The small cytoplasmic chaperones, 16.9 kDa class I heat shock protein 3 and heat shock protein18c were also up regulated. Other ER enzymes involved in the oxidation of cysteine to form disulfide bonds including protein disulfide isomerase (PDI) and ER Membrane-Localized Oxidoreductase 1 (ERO1) were increased (Onda et al., 2009). These genes are related to the unfolded protein response and their up regulation is likely due to alteration of protein body structure in the ER (Hunter et al., 2002).

Stress-response and defense genes up regulated in W64A*o2* included the following: alliin lyase 2 (alliinase) and cystatin 6, which are part of the defense response against herbivores (Kuettner et al., 2002; Martinez et al., 2009); xylanase inhibitor protein 1 and glycine–aspartic acid–serine-leucine (GDSL)-motif lipase/hydrolase-like protein, both of which are involved in the defense against fungal pathogens (Igawa et al., 2004; Oh et al., 2005); and a Pi starvation-induced protein and an ABA-responsive 40 kDa protein (Moons et al., 1995; Mukatira et al., 2001; Vierling, 1991). A MAP kinase was up regulated, as were several MAP kinase responsive genes. These include the respiratory burst oxidase protein, homolog B (RBOHB), and an inducible form of the NADPH oxidase, a downstream effector in the mitogen-activated protein kinase (MAPK) regulated signaling

pathway that generates reactive oxygen species (ROS) and triggers innate immunity in response to various stresses (Asai et al., 2008). Additionally, the WRKY transcription factor was up regulated, which is phosphorylated and activated by MAPKs in response to biotic and abiotic stresses (Pandey and Somssich, 2009). These up regulated stress responses are unlikely to confer enhanced resistance to pests and most likely represent pleiotropic responses to mutation of *o2*, because there is ample evidence that *o2* is much more susceptible to pests.

*Proteomic Comparison of opaque2 and Wild Type Lines*

In order to detect differences in non-zein protein accumulation in W64A*o2* and wild type lines, we performed 2D SDS-PAGE analysis with equal amounts of non-zein proteins purified from mature endosperms using a borate extraction method (Lending and Larkins, 1989). Mature kernels were analyzed in order to determine if abundant non-zein proteins that contribute to increased lysine were consistent with their gene expression during endosperm development. After visualization and alignment of gels, 40 protein spots that were differentially resolved or showed altered accumulation levels were excised from gels for identification (Figure 2.2). Proteins of interest were identified by MALDI-TOF peptide mass mapping of trypsin digests of the protein spots. GBSSI, enolase 1, legumin-like protein, GAPDH, TIM and SDH showed increased accumulation in o2, while enolase 2 and HSP3 showed no alteration in accumulation (Table 2.1). Many of the largest differences in protein accumulation were reflected in the transcript levels measured by transcript profiling at 22 DAP. The exceptions were GBSSI and enolase 1, which had inconsistent fold-changes in multiple studies (Frizzi et al., 2010; Hartings et al., 2011; Hunter et al., 2002). This could be due to differences in genetic backgrounds of the lines

analyzed or the different environments in which the materials were grown. However, in the W64A background the transcript profiling and proteomic data showed enolase 1 accumulation was higher in *o2* (Figure 2.2). Notably, there was a significant increase (~1.8-fold) in the accumulation of GAPDH, which contains over 8% lysine, but was not found to be significantly different in transcript abundance in the transcript profiling data. Likewise there was a >2.5-fold increase in some SDH1 isoforms and this protein contains 4% lysine, which is lower than GAPDH, but nearly two-fold higher than the typical total lysine content of wild type maize endosperm. The increase in GAPDH and SDH1 could contribute significantly to the elevated level of lysine in W64A*o2*, and the expression of these very abundant proteins may be associated with the expression of translation elongation factor 1A (EFIA), which is correlated with lysine content in maize endosperm but is not sufficient to explain the total increase in lysine content (Habben et al., 1995).

*Validation of Gene Expression*

Quantitative real-time polymerase chain reaction (qRT-PCR) was performed for several genes encoding both up and down regulated transcripts in W64A*o2* to validate the results from the transcript profiling experiment with endosperms from both genotypes at 22 DAP. Retinoblastoma-related protein 1 (RRB1) was used as the reference gene, because it is consistently expressed in both genotypes (see Materials and Methods). The RIP gene, b-32, was chosen as a positive control for qRT-PCR, because it is known to be down regulated in *o2* mutants (Bass et al., 1992). Genes were selected based on the following categories: 1) genes with expression that was highly reduced in the *o2* mutant in

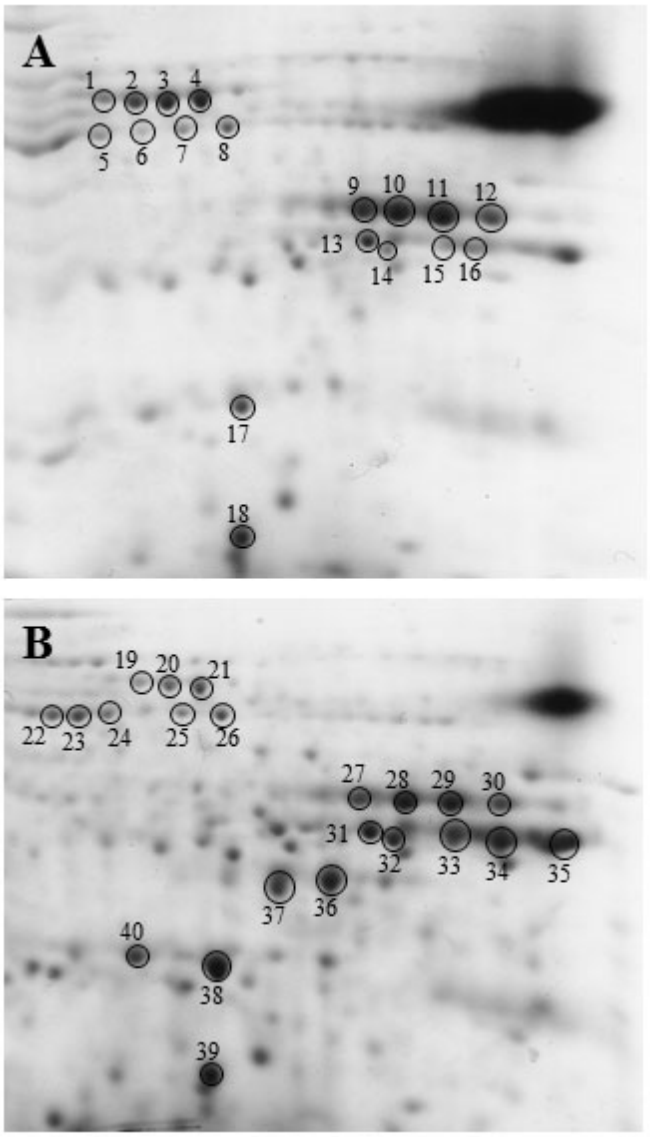


Figure 2.2: 2D SDS-PAGE analysis of W64A + and W64A*o2*.Non-zein proteins from W64A + (A) and W64A*o2* (B) lines were extracted from mature endosperm flour and separated by 2D SDS-PAGE. Circled, numbered spots were excised from gels and protein identities were determined by MALDI-TOF peptide mass mapping and correspond to the rows in Table 2.1.

Table 2.1: Identity and expression differences of proteins separated by 2D SDS-page. *Italicized values indicate significant difference by ANOVA ($p < 0.05$; $n = 3$).

| Spot | Genbank | Annotation | Fold-change |
|---|---|---|---|
| 1 | P04713 | Granule-bound starch synthase 1 | |
| 2 | P04713 | Granule-bound starch synthase 1 | |
| 3 | P04713 | Granule-bound starch synthase 1 | |
| 4 | P04713 | Granule-bound starch synthase 1 | |
| 5 | NP_001105896 | enolase 1 | |
| 6 | NP_001105896 | enolase 1 | |
| 7 | NP_001105371 | enolase 2 | |
| 8 | NP_001105371 | enolase 2 | |
| 9 | BAB11045 | sorbitol dehydrogenase-like protein | |
| 10 | BAB11045 | sorbitol dehydrogenase-like protein | |
| 11 | BAB11045 | sorbitol dehydrogenase-like protein | |
| 12 | BAB11045 | sorbitol dehydrogenase-like protein | |
| 13 | ACG32147 | eukaryotic translation initiation factor 2 alpha | |
| 14 | AAO63267 | Legumin-like protein, complete | |
| 15 | Q43247 | Glyceraldehyde-3-phosphate dehydrogenase | |
| 16 | Q43247 | Glyceraldehyde-3-phosphate dehydrogenase | |
| 17 | NP_001140424 | triosephosphate isomerase | |
| 18 | ACG35098 | 17.4 kDa class I heat shock protein 3 | |
| 19 | P04713 | Granule-bound starch synthase 1 | *−2.5** |
| 20 | P04713 | Granule-bound starch synthase 1 | *−2.1* |
| 21 | P04713 | Granule-bound starch synthase 1 | −1.5 |
| 22 | NP_001105896 | enolase 1 | 1.3 |
| 23 | NP_001105896 | enolase 1 | 1.2 |
| 24 | NP_001105896 | enolase 1 | 1.1 |
| 25 | NP_001105371 | enolase 2 | 1.1 |
| 26 | NP_001105371 | enolase 2 | 1.3 |
| 27 | BAB11045 | sorbitol dehydrogenase-like protein | −1.5 |
| 28 | BAB11045 | sorbitol dehydrogenase-like protein | −1.2 |
| 29 | BAB11045 | sorbitol dehydrogenase-like protein | *−1.4* |
| 30 | BAB11045 | sorbitol dehydrogenase-like protein | −1.2 |
| 31 | ACG32147 | eukaryotic translation initiation factor 2 alpha | 1.2 |
| 32 | AAO63267 | Legumin-like protein, complete | 1.5 |
| 33 | Q43247 | Glyceraldehyde-3-phosphate dehydrogenase | *1.7* |
| 34 | Q43247 | Glyceraldehyde-3-phosphate dehydrogenase | *1.8* |
| 35 | AAA87580 | Glyceroldehyde-3-phosphate dehydrogenase | 1.6 |
| 36 | NP_001149440 | sorbitol dehydrogenase homolog1 | *2.7* |
| 37 | ABA70761 | sorbitol dehydrogenase | *2.9* |
| 38 | NP_001140424 | triosephosphate isomerase, cytosolic | 1.5 |
| 39 | ACG35098 | 17.4 kDa class I heat shock protein 3 | 1.2 |
| 40 | NP_001140424 | triosephosphate isomerase, cytosolic | 1.6 |

the profiling experiment, such as proteosome regulatory subunit AAA-ATPase (AAA-ATPase), stem-specific protein (TSJT1), 16-kDa oleosin, CI-1B and BGAF, which had not been characterized in previous studies; 2) starch biosynthesis genes, such as Zpu1, starch branching enzyme IIb (BEIIb) and GBSSI; 3) genes related to carbohydrate metabolism that were changed in the transcript profiling or 2D SDS-PAGE analysis, including GAPDH, sorbitol dehydrogenase 1 (SDH1), TIM, enolase 1 and PGK; and 4) other genes that showed changes in W64A*o2* according to profiling results, such as actin2, legumin1, 17.4 kDa class I heat shock protein 3 (HSP3) and LKR-SDH1.

The relative expression levels of transcripts among various samples in the qRT-PCR generally agreed with the profiling results (Pearson correlation coefficient $r = 0.80$, ANOVA $p < 0.001$; supplemental Figure S2.1). However, in some cases the transcript fold-changes measured by Gene Calling were higher than those from qRT-PCR. The transcript level of the known O2-regulated gene b-32 was significantly reduced in *o2* by both transcript profiling and qRT-PCR (Figures 2.3A and 2.3B), consistent with previous reports (Hartings et al., 2011; Hunter et al., 2002). The transcript levels of BGAF, 16 kDa oleosin, CI-1B, TSJT1 and AAA-ATPase were all significantly reduced in *o2*, compared to W64A + by qRT-PCR (Figure 2.3B) and Gene Calling (Figure 2.3A and supplemental Table S2.1). qRT-PCR analysis also confirmed the expression of starch synthesis genes that were found to be up regulated in o2 such as Zpu1 and BEIIb (Frizzi et al., 2010; Hunter et al., 2002) (Figure 2.3). Although in the case of Zpu1, the increase was greater in the GeneCalling results (2.27-fold versus 1.55-fold in qRT-PCR). These results indicate that the majority of changes in gene expression are the result of the *o2* mutation, and not the genetic background or environmental conditions in the field. On the other hand, the

expression of some genes was not consistent, notably LKR-SDH and GBSSI. As stated previously LKR-SDH was not significantly different in the transcript profiling data (Figure 2.3A), yet when measured by qRT-PCR in individuals grown in a different environment there was a significant difference (Figure 2.3B). Likewise, the difference in the transcript level of GBSSI was not consistent among previous reports (Frizzi et al., 2010; Hartings et al., 2011; Hunter et al., 2002). GBSSI transcript increased in *o2* according to the GeneCalling analysis, but decreased significantly based on the qRT-PCR analysis. These results together with data from prior studies indicate that expression of GBSSI and LKR-SDH may be dependent on both genotype and environmental conditions.

Carbohydrate metabolism-related genes significantly affected in *o2* by either GeneCalling or 2D SDS-PAGE analysis were also examined by qRT-PCR. Of the genes that were tested, only SDH1 showed significantly higher expression in *o2* at 22 DAP (Figure 2.3). Finally, the expression of HSP3 and actin2 measured by qRT-PCR did not agree with the GeneCalling results, but the decreased level of actin2 in *o2* was observed in other experiments (Hartings et al., 2011; Hunter et al., 2002). There is a possibility that this difference was due to primer specificity, since both HSP and actin belong to multigene families and there are other family members that share significant sequence similarity. Therefore, multiple gene family members could be detected at the same time in qRT-PCR.

*Western Blot Analysis of opaque2 and Wild Type Lines*

Western blot analysis of 22 DAP W64A + and W64A*o2* maize endosperm was performed to extend the transcript profiling and proteomic analysis (Figure 2.4); quantitative measurement by densitometry is shown in Table 2.2. In contrast to the gene

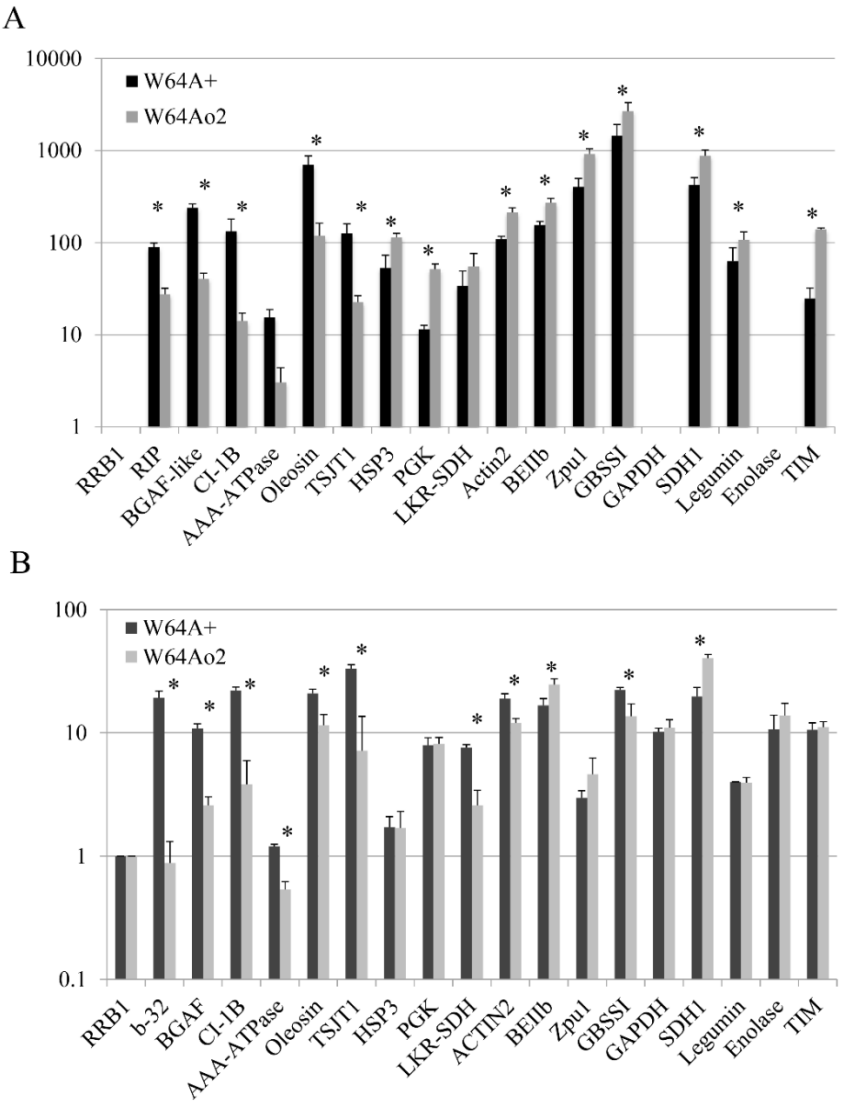


Figure 2.3: Confirmation of genes or proteins altered in W64Ao2 by qRT-PCR. Expression of the indicated genes was analyzed in 22 DAP endosperms of W64A + and W64Ao2 by GeneCalling transcript profiling (A) or qRT-PCR to confirm the difference in expression in the transcript profiling, or to measure the expression levels of genes that were differentially expressed by 2D SDS-PAGE analysis (B). Asterisks indicate significantly different expression using the two-tailed t-test at a level of $p < 0.05$. Note that the Y-axis is logarithmic to accommodate the wide differences in gene expression levels among the transcripts. Missing columns in A indicate that the gene was not among the genes that had a confirmed identity in the transcript profiling data.

expression data, the *o2* mutants showed an increase in GAPDH protein abundance by both 2D SDS-PAGE and by western blot analysis. This suggests that the GAPDH protein may

be particularly stable in endosperm cells and therefore accumulates to a substantially higher level than indicated by its transcript abundance during seed development. Although expression of actin2 was increased in *o2* in the transcript profiling data, no measurable protein difference was observed on western blots (Figure 2.4). However, the anti-actin antibody available is reported to recognize many isoforms of the protein across multiple kingdoms. Therefore it was not specific for the product of the gene that was up regulated in the present analysis. As expected, EF1A was significantly higher in *o2*, whereas other translation-related factors were either slightly higher (translation initiation factor 5A, IF5A) or slightly lower (ribosomal protein S6, S6RP) in W64A*o2*. However, there were no measurable differences in eukaryotic translation initiation factor 4G (eIF4G), eukaryotic translation initiation factor 2 alpha subunit (eIF2α, or eukaryotic translation initiation factor 4E (eIF4E) (not shown). Analysis of starch biosynthetic enzymes showed that BEIIa and BEIIb were not different between *o2* and wild type. However, there was increased accumulation of starch synthase IIa (SSIIa) and starch branching enzyme I (BEI) in *o2*. Both of these enzymes have significant effects on starch structure when mutated or knocked down by RNAi, which results in the accumulation of amylopectin with relatively short glucan chains (Nakamura, 2002; Zhang et al., 2004). In contrast, BEI preferentially produced longer chain length branches (>16) compared with BEIIb, which preferentially produced shorter branches (<12) in an in vitro assay (Guan et al., 1997). Together, these observations suggest that in the W64A*o2* mutant the average chain length of amylopectin branches would be greater than in W64A+.

*Analysis of Starch*

The expression of several starch biosynthesis genes varied between W64A + and W64A*o2* based on gene expression analysis and 2D SDS-PAGE. Interestingly, *o2* was the only mutant among eight different isogenic opaque mutant lines that showed significant expression differences in starch biosynthesis genes (Hunter et al., 2002, Gibbon and Larkins unpublished). Because levels of several starch biosynthesis enzymes were altered in *o2*, SSIIa and BEI in particular, the properties of the starch from W64A + and W64A*o2* were analyzed by differential scanning calorimetry (DSC) to determine if these changes affected the starch structure. The onset and peak endotherm temperatures as well as the total enthalpy of gelatinization were significantly higher for W64A*o2* (Table 2.3). The higher values for these thermal properties in *o2* are consistent with starch that has longer amylopectin branches and higher crystalline starch content. To further characterize the structure of the starch, the amylopectin branch length distributions of W64A + and W64A*o2* were measured. Debranched starch glucans were separated by capillary electrophoresis and the resulting branch length distributions were compared (Figure 2.5). The two genotypes had similar molar percent content of glucans, but the distribution of glucans from W64A*o2* was shifted toward a higher degree of polymerization (Figure 2.5A). A difference plot clearly showed a marked increase in glucan chains with a degree of polymerization between 15 and 25 glucose subunits in W64A*o2* (Figure 2.5B). These results were similar to what was previously observed for o2 in the CM105 inbred line (Gibbon et al., 2003). Together, the western blot analysis and analysis of starch structure suggest that enhanced BEI or SSIIa activity results in amylopectin with significantly longer glucan chains in W64A*o2*. These changes in the crystallinity and branching pattern of

W64A*o2* starch may alter the association of the starch granules with endosperm proteins and thus promote formation of a soft, opaque

phenotype.

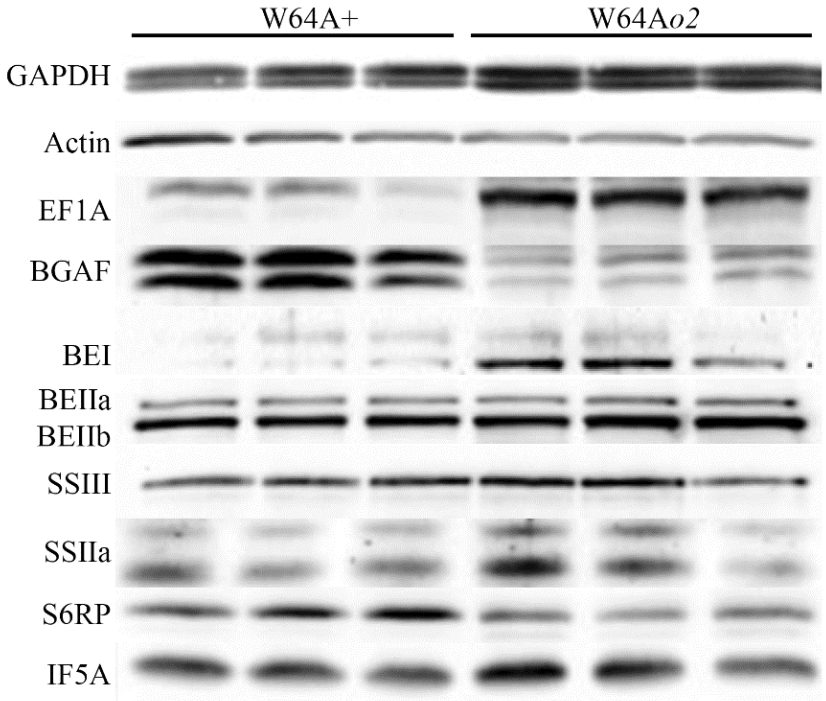


Figure 2.4: Western blot analysis of selected proteins in W64A + and W64A*o2*. Western blots were performed using antisera against the proteins indicated on the left. Three replicate samples for each line were obtained from independent ears frozen at 22 DAP and 25 µg of protein from each was separated by SDS-PAGE followed by western blotting. Each band was analyzed by densitometry and the fold change values calculated for each protein and the values are presented in Table 2.2.

*Discussion*

The analysis of *opaque2* transcription patterns by Gene Calling significantly expanded the results of previous studies using microarrays, and by combining transcript profiling with proteomic analysis, we were able to document the presence of certain

Table 2 2: Densitometry analysis of western blots of wild type and *opaque2* endosperm extracts. *p-value for two tailed Student's t-test. Italicized figures are significantly different at a level of $p < 0.05$ (n = 3).

| | W64A+ | | W64A*o2* | | | |
|---|---|---|---|---|---|---|
| Protein | Mean | SD | Mean | SD | Fold-Change | p* |
| GAPDH | 9.80 | 0.65 | 14.73 | 0.94 | 1.50 | *0.002* |
| EF1A | 2.57 | 0.82 | 7.19 | 0.62 | 2.79 | *0.001* |
| S6RP | 7.16 | 0.59 | 5.03 | 0.91 | −1.43 | *0.03* |
| IF5A | 8.19 | 0.88 | 9.37 | 1.61 | 1.14 | 0.33 |
| Actin | 2.84 | 0.78 | 2.47 | 0.23 | −1.15 | 0.47 |
| BGAF | 7.55 | 1.62 | 2.23 | 0.38 | −3.38 | *0.005* |
| SSIIa | 2.68 | 0.43 | 3.74 | 0.87 | 1.40 | 0.13 |
| SSIII | 5.22 | 0.79 | 6.35 | 0.84 | 1.22 | 0.17 |
| BEI | 0.69 | 0.07 | 2.04 | 0.54 | 2.97 | *0.01* |
| BEIIa | 3.08 | 0.17 | 3.74 | 0.67 | 1.21 | 0.18 |
| BEIIb | 5.83 | 0.64 | 7.52 | 1.02 | 1.29 | 0.07 |

Table 2.3: DSC analysis of W64A + and W64A*o2* starch. P values were calculated by student's t-test.

| | W64A+ | W64A*o2* | p |
|---|---|---|---|
| Onset (°C) | 68.02 ± 0.659 | 71.38 ± 0.169 | <.0001 |
| Peak Endotherm (°C) | 71.84 ± 0.846 | 74.65 ± 0.172 | <.0001 |
| Total Enthalpy (J/G) | 11.29 ± 0.83 | 14.39 ± 1.00 | <.0001 |

abundant lysine-containing proteins related to primary carbon metabolism. This is consistent with prior proteomic analyses of developing kernels (Damerval and Le Guilloux, 1998), but the relative levels appear to be proportionally much higher in mature kernels. The two proteins that appeared to be most abundant were SDH1 and GAPDH, which have lysine contents of 4.2 and 8.5 percent, respectively. Especially for GAPDH, its relatively high accumulation in mature endosperm could contribute a substantial proportion of the total increased lysine observed in *o2*. The results could explain the relatively high lysine

content of W64A*o2* endosperm, and the basis of the phenotypic variability for this trait among maize inbreds (Moro et al., 1996).

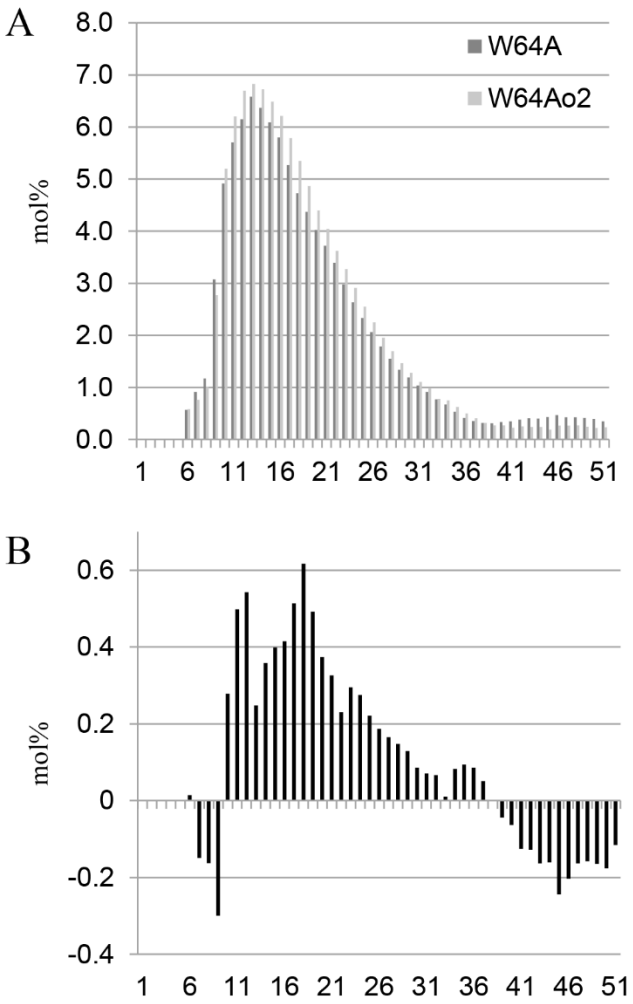


Figure 2.5: Amylopectin branch length analysis. Solubilized starch was de-branched in the presence of isoamylase and the resulting glucan chains were separated by capillary electrophoresis. (A) Histograms of the distribution of glucan chains were similar but W64A*o2* was shifted toward a higher degree of polymerization. (B) Difference plot was calculated by subtracting the W64A + values from the W64A*o2* values showed a substantial increase in chains with a degree of polymerization between 15 and 25 glucose subunits. The histograms represent the average of three replicates for each genotype.

Additional genes that contribute to the deleterious phenotypes of *o2* and that appear to be related to pest resistance were identified in this analysis. RIP is a well-known *o2* target gene and plays a role in the defense against fungal pathogenesis (Ferreira et al., 2007). Likewise, BGAF was strongly down regulated, and it is suggested to have a role of concentrating beta-glucosidase at wound sites to promote activation of glycosylated defense compounds (Blanchard et al., 2001). Other down regulated defense proteins included Cl-1B, BAP2, and defensin SD2. Down regulation of such defense proteins may synergistically contribute to the high susceptibility of *o2* to fungal and insect pests. Investigation of these genes in *o2* or modified *o2* backgrounds may aid in the development of better performing high lysine maize lines.

Finally, *o2* was the only opaque mutant to show significant alteration of starch biosynthetic gene expression. In particular, the up regulation of BEI and/or SSIIa appears to explain the production of starch granules that are more highly crystalline in character, which could contribute to the opaque phenotype. Former studies indicated that an alteration in starch granule structure could be an important contributor to the restoration of vitreous endosperm by *o2* modifiers in QPM (Gibbon et al., 2003). Our recent studies indicate that pullulanase activity is significantly higher in QPM and correlates well with the extent of endosperm modification, and this change is most likely due to a reduction in glucan chain length relative to soft *o2* mutants (Wu and Gibbon unpublished data). Therefore, manipulation of starch quality by transgenic means or naturally occurring alleles of BEI or SSIIa may be a way to enhance kernel quality and suppress the opaque phenotype for the improvement of QPM or other high lysine maize lines.

*Authors' Contributions*

MJ Experimental data (Tables 2.1 and 2.2. Figures 2.3 and 2.4. Supplemental Tables S2.1 and 2.2. Supplemental Figure S2.1) and writing. KC Experimental data (Table 3). HW Experimental data (Figure 5). RJ Experimental design and data (Supplemental File 1, included in this dissertation with permission). BAL Writing and experimental design. BCG Writing, experimental design and data (Figures 2.1 and 2.2, included in this dissertation with permission). All authors read and approved the final manuscript.

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*Supplemental Data*

Supplemental File 2.1: Sequence tags used to determine gene identities. FASTA formatted sequence text file. The sequences for each band that were confirmed by either competitive PCR or sequencing of the band were used to determine gene identitiy by searching the non-redundant genbank database using the basic local alignment and search tools BLASTN and BLASTX.

>b0w0-179.8
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TGGGTTGGATGCAAGCTT

Supplemental Table S2.1: qRT-PCR primer sequences.

| Gene name | Short name | Forward primer 5' - 3' | Reverse primer 5' - 3' |
|---|---|---|---|
| Zea mays retinoblastoma-related protein 1 | RRb1 | GCTGTTTCTGGTTATGTCTGTCCT | CTTTTGAGTACTTCTGTGCCTGAC |
| glyceraldehyde 3-phosphate dehydrogenase | GAPDH | GCGGATCCACTGTCGATGTTTCTG TTGTTGATCTAAC | GCCCATGGTTACTTGGTGCTGTTC ATGTGGCGG |
| Zea mays ribosome inactivating protein | RIP | ACGACCTGGCGAAGAAGAAG | CACACCATGACCACCAGCTT |
| β-glucosidase-aggregating factor | BGAF | CGACACAGGTGGTCAGACG | GGTGCTGGTGACAATCCTGA |
| Zea mays sorbitol dehydrogenase homolog1 | SDH1 | GGTTCAGCAAGACCATGTCG | GCCAGGTGTCCTTGTACCG |
| Zea mays legumin-like protein | Legumin | GCTTCCATGTCGTGTCCAAG | CTTGGAGCGGAACAGCTTCT |
| Zea mays enolase | Enolase | GGGTCACATGCTGGAAACAA | CCACCTTCATCCCCAACATT |
| 17.4 kDa class I heat shock protein 3 | HSP3 | CAGCAGTCTCTTCCCCTCGT | TCCGTCTTCTCCTCCTGCTC |
| Zea mays triosephosphate isomerase, cytosolic | TIM | CTGGGTGAAGAAGGGAGGTG | AGACAGGGCATACGCAACCT |
| Zea mays granule-bound starch synthase 1 | GBSS1 | GAGGACGTCGTGTTCGTCTG | CAGTTGATCTTCCGGCCTTC |
| Zea mays stem-specific protein TSJT1 | TSJT1 | GTAGGCCTCGATGACCAGGA | CTACTCCCACGCCAACCAG |
| Proteosome regulatory subunit AAA-ATPase | AAA-ATPase | AAGACCTGAGGCAGGCAAAG | CTGCCCAGCTCTGTTCCTCT |
| oleosin 16-kDa | Oleosin | ACGTCAAGGATGCAGCACAG | CCAGACACATGCATGCACAC |
| subtilisin-chymotrypsin inhibitor CI-1B | CI-1B | ACTTTCGCCCTGATCGTGTT | TAATTTCTTGGGGCCCCTTT |
| Actin2 | Actin2 | CCAAGCAGCATGAAGATCAA | ATGCAAATCTGCCACACGTA |
| Zea mays starch-branching enzyme IIb | BEIIb | CCGACGCTGGACTATTTGGT | GCAACGAGTACCCCGCTATC |
| Zea mays pullulanase-type starch debranching enzyme1 | Zpu1 | GCTTCAGTTGCATCCAGTGC | TGGGCAACGTCTTATTCAATC |
| Zea mays cytosolic phosphoglycerate kinase | PGK | CCCCATGGGAGTGTTTGAGT | CAAAGCTGGCAGAACCTGAG |

Supplemental Table S2.2: Gene expression values for differentially expressed bands with confirmed sequences. *Expression values are fold-change. Negative values indicate increased expression in W64A*o2* relative to W64A wild type.

| Band ID | Identity/Top BLAST hit | Relative expression* | GO Biological Process |
|---|---|---|---|
| l0r0-176.5 | GDSL-motif lipase/hydrolase-like protein [Zea mays] | -42.94 | oil metabolism |
| r0k0-378.2 | Putative retrotransposon protein [Zea mays] | -7.57 | carbohydrate metabolism |
| u0w0-144.3 | Unknown | -6.65 | unknown |
| u0w0-247.4 | Triosephosphate isomerase, cytosolic [Zea mays] | -5.46 | carbohydrate metabolism |
| m1l0-250.7 | Phenylcoumaran benzylic ether reductase -like protein [Populus trichocarpa] | -5.25 | lignan biosynthesis |
| m1a0-73.6 | 19kD β-zein | -4.71 | nutrition reservoir |
| s0w0-342.8 | Phosphoglycerate kinase, cytosolic [Zea mays] | -4.02 | carbohydrate metabolism |
| l0n0-46.1 | Unknown | -4.02 | Unknown |
| m1g1-126.6 | Mannose-1-phosphate guanyltransferase [Zea mays] | -3.83 | carbohydrate metabolism |
| u0f0-75.4 | 16kD zein | -3.7 | nutrition reservoir |
| u0w0-263.8 | Aspartic proteinase oryzasin-1 [Zea mays] | -3.33 | protein turnover |
| i0c0-41.5 | Coatomer subunit gamma | -2.96 | Transport |
| m0v0-122.7 | Cystatin6 [Zea mays] | -2.9 | defense response |
| m1e1-262.5 | Alanine aminotransferase 2 [Zea mays] | -2.87 | amino acid metabolism |
| s0w0-364.5 | Alcohol dehydrogenase 1 (ADH1) | -2.86 | carbohydrate metabolism |
| m1a0-180.7 | 22kD zein | -2.68 | nutrition reservoir |
| d0g0-203.9 | Nuclear protein [Zea mays] | -2.61 | Unknown |

Supplemental Table S2.2, Continued

| Band ID | Identity/Top BLAST hit | Relative expression* | GO Biological Process |
|---|---|---|---|
| u0w0-166.5 | DNA-directed RNA polymerases I, II, and III 17.1 kDa polypeptide [Zea mays] | -2.55 | transcription/translation |
| d0g0-241.4 | Farnesyl pyrophosphate synthase [Zea mays] | -2.45 | lipid metabolism |
| g1n0-446.0 | Rhodopsin-like receptor [Zea mays] | -2.39 | signal transduction |
| u0v0-293.2 | CHITINASE B | -2.38 | carbohydrate metabolism |
| i0l0-390.0 | Protein disulfide isomerase [Zea mays] | -2.37 | protein folding |
| u0g1-218.4 | Putative VP2-like RING Finger TF \| VIP2 protein [Avena fatua] | -2.35 | transcription/translation |
| r0k0-193.9 | Pullulanase-type starch debranching enzyme1 [Zea mays] | -2.27 | carbohydrate metabolism |
| g1n0-261.1 | 2PGK 2-phosphoglycerate kinase [Arabidopsis thaliana] | -2.25 | carbohydrate metabolism |
| i0r0-82.0 | 16.9 kDa class I heat shock protein 3 [Zea mays] | -2.22 | stress response/defense response |
| g0m0-51.6 | ZIP zinc/iron transport family protein [Zea mays] | -2.21 | Transport |
| l0r0-89.8 | Pol protein homolog - maize retrotransposon | -2.19 | Unknown |
| i0n0-159.3 | Oryza sativa GDP dissociation inhibitor protein OsGDI1 | -2.16 | signal transduction |
| l0r0-186.0 | Respiratory burst oxidase protein B [Zea mays] | -2.15 | oxidation-reduction process/defense response |
| u0e1-199.0 | Sorbitol dehydrogenase homolog1 [Zea mays] | -2.09 | carbohydrate metabolism |
| i0n0-200.4 | Calnexin [Zea mays] | -2.08 | protein folding |

Supplemental Table S2.2, Continued

| Band ID | Identity/Top BLAST hit | Relative expression* | GO Biological Process |
|---|---|---|---|
| m1a0-110.4 | Actin | -1.99 | Cytoskeleton |
| g1n0-284.0 | WRKY17 transcription factor [Triticum aestivum] | -1.97 | transcription/translation |
| l0r0-318.4 | Unknown | -1.91 | Unknown |
| u0g1-173.2 | MAP kinase [Zea mays] | -1.9 | signal transduction |
| d0g0-384.4 | Epoxide hydrolase 2 [Zea mays] | -1.87 | catalytic activity |
| l0u0-78.0 | Fructokinase-1 [Zea mays] | -1.85 | carbohydrate metabolism |
| i0l0-124.9 | Granule-bound starch synthase 1 [Zea mays] | -1.84 | carbohydrate metabolism |
| u0g1-418.8 | Calreticulin2 [Zea mays] | -1.83 | protein folding |
| i0r0-116.8 | NAM-related protein 1 [Zea mays] | -1.83 | transcription/translation |
| m1w0-162.3 | 19KD zein | -1.81 | nutrition reservoir |
| u0e1-53.0 | Pi starvation-induced protein [Zea mays]. | -1.8 | defense response |
| l0n0-236.7 | Unknown | -1.8 | Unknown |
| m1l0-408.1 | Mitochondrial 2-oxoglutarate/malate carrier protein [Zea mays] | -1.78 | Transport |
| l0n0-122.1 | RING-H2 finger protein ATL1R [Zea mays] | -1.76 | transcription/translation |
| l0u0-281.0 | Grancalcin [Zea mays] | -1.75 | signal transduction |
| u0g1-84.4 | 60S ribosomal protein L33-B [Zea mays] | -1.75 | transcription/translation |
| d0g0-209.4 | 1,4-alpha-glucan-branching enzyme 2, chloroplastic/amyloplastic precursor [Zea mays] | -1.74 | carbohydrate metabolism |

Supplemental Table S2.2, Continued

| Band ID | Identity/Top BLAST hit | Relative expression* | GO Biological Process |
|---|---|---|---|
| m1n0-168.8 | Protein transport protein SEC31 [Arabidopsis thaliana] | -1.74 | Transport |
| u0e1-278.1 | Globulin precursor [Zea mays] | -1.73 | nutrition reservoir |
| i0n0-209.1 | Protein kinase-like domain containing protein [Oryza sativa] | -1.7 | signal transduction |
| u0v0-41.2 | Legumin 1 [Zea mays] | -1.69 | nutrition reservoir |
| r0s0-183.7 | Ethylene receptor homolog2 [Zea mays] | -1.67 | signal transduction |
| m1a0-228.5 | 27kD γ-zein | -1.66 | nutrition reservoir |
| r0a0-209.1 | Osr40g2 [Oryza sativa] | -1.63 | defense response |
| m1w0-94.5 | Zea mays heat shock protein18c (hsp18c) | -1.63 | defense response |
| m1a0-153.9 | Chaperone DNA J2 [Zea mays] | -1.61 | protein folding |
| l0m0-197.2 | Xylanase inhibitor protein 1 [Zea mays] | -1.59 | defense response |
| g1n0-213.8 | F-box protein FBL2 [Zea mays] | -1.57 | protein turnover |
| l0e1-164.1 | Unknown | -1.57 | Unknown |
| m1e1-92.0 | Alliin lyase 2 [Zea mays] | -1.53 | defense response |
| l0n0-82.6 | Trehalose 6 phosphate synthase | -1.52 | secondary metabolism |
| m0r0-95.0 | Unknown | -1.52 | Unknown |
| f0i0-327.7 | Symplekin [Arabidopsis thaliana] | -1.5 | transcription/translation |
| d0l0-411.9 | Vacuolar sorting receptor 1 precursor [Zea mays] | -1.5 | Transport |
| i0r0-186.8 | ERO1 | -1.48 | oxidation reduction |
| u0e1-147.7 | Alpha globulin [Zea mays] | -1.39 | nutrition reservoir |
| i0l0-368.8 | set1 complex component swd2 [Zea mays] | -1.35 | signal transduction |

Supplemental Table S2.2, Continued

| Band ID | Identity/Top BLAST hit | Relative expression* | GO Biological Process |
|---|---|---|---|
| l0r0-240.1 | RNA binding protein Rp120 [Oryza sativa Japonica Group] | -1.33 | transcription/translation |
| i0u0-149.5 | 17.4 kDa class I heat shock protein 3 [Zea mays] | 1.16 | defense response |
| r0a0-136.7 | Acyl-CoA binding protein [Zea mays] | 1.54 | oil metabolism |
| l0r0-126.8 | Unknown | 1.54 | Unknown |
| i0a0-180.4 | 40S ribosomal protein S3a [Zea mays] | 1.6 | transcription/translation |
| u0e1-281.6 | 27kD γ-zein | 1.62 | nutrition reservoir |
| m1a0-254.1 | 22kD zein | 1.62 | nutrition reservoir |
| i0u0-342.7 | Unknown | 1.68 | Unknown |
| n0s0-143.3 | Gamma-interferon-inducible lysosomal thiol reductase [Zea mays] | 1.7 | catalytic activity |
| l0n0-252.8 | 60S ribosomal protein L19-3 [Zea mays] | 1.72 | transcription/translation |
| i0a0-267.0 | Unknown | 1.72 | Unknown |
| r0a0-306.9 | 27kD γ-zein | 1.74 | nutrition reservoir |
| i0q0-74.7 | Omega-6 fatty acid desaturase, endoplasmic reticulum isozyme 2 [Zea mays] | 1.74 | oil metabolism |
| n0s0-44.1 | Brassinosteroid biosynthesis-like protein [Zea mays] | 1.8 | sterol metabolism |
| u0g1-105.5 | 19kD D zein | 1.81 | nutrition reservoir |
| d0l0-156.0 | Ketol-acid reductoisomerase [Zea mays] | 1.85 | amino acid metabolism |
| m1g1-303.2 | 19kD D zein | 1.9 | nutrition reservoir |
| u0v0-116.1 | 27kD γ-zein | 1.94 | nutrition reservoir |
| n0s0-200.4 | Chorismate mutase [Zea mays] | 1.94 | secondary metabolism |
| i0u0-181.8 | Basal layer antifungal protein2 [Zea mays] | 1.94 | defense response |

Supplemental Table S2.2, Continued

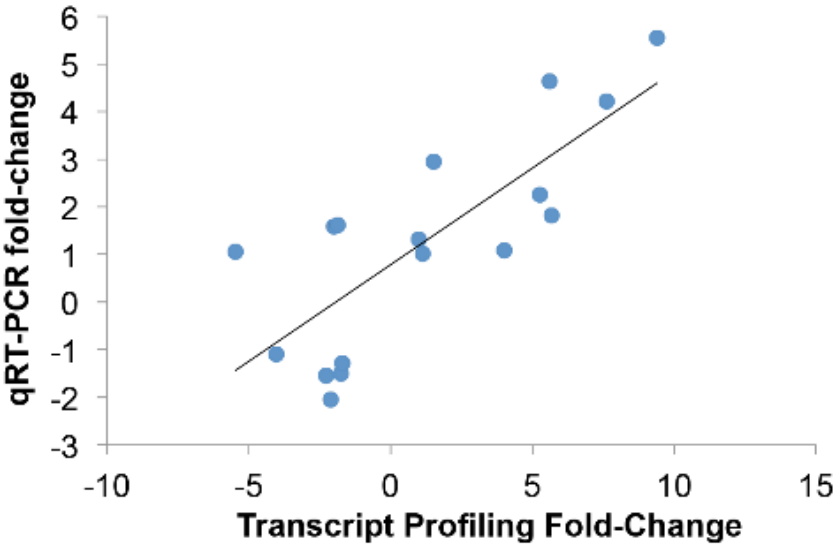
| Band ID | Identity/Top BLAST hit | Relative expression* | GO Biological Process |
|---|---|---|---|
| m1n0-361.7 | Pyruvate dehydrogenase E1 alpha subunit [Zea mays] | 1.95 | carbohydrate metabolism |
| u0e1-267.8 | Oleosin 18 kDa [Zea mays] | 1.96 | nutrition reservoir |
| i0l0-411.0 | Dehydration-responsive protein RD22 [Prunus persica] | 2.01 | defense response |
| m1e1-112.2 | 19KD zein | 2.07 | nutrition reservoir |
| w0c0-127.0 | ERF-like protein [Zea mays] | 2.07 | transcription/translation |
| m1l0-86.4 | 18S ribosomal RNA gene | 2.07 | transcription/translation |
| i0a0-145.9 | Miniature seed1 [Zea mays] | 2.18 | carbohydrate metabolism |
| u0e1-260.3 | 27kD γ-zein | 2.19 | nutrition reservoir |
| d0v0-156.9 | 27kD γ-zein | 2.2 | nutrition reservoir |
| w0c0-71.6 | Probable non-specific lipid-transfer protein 2 (LTP 2) [Zea mays] | 2.22 | Transport |
| n0s0-146.7 | NAC domain-containing protein 48 [Zea mays] | 2.28 | transcription/translation |
| d0v0-87.8 | Katanin p60 ATPase-containing subunit [Zea mays] | 2.41 | Cytoskeleton |
| i0u0-49.4 | Flavin monooxygenase [Zea mays] | 2.41 | secondary metabolism |
| i0q0-71.9 | Alanine-glyoxylate aminotransferase 2 [Zea mays] | 2.44 | amino acid metabolism |
| f0i0-181.3 | 19kD D zein | 2.46 | nutrition reservoir |
| s0w0-228.1 | 19KD zein | 2.51 | nutrition reservoir |
| m1a0-183.7 | 22kD zein | 2.52 | nutrition reservoir |
| l0n0-327.7 | Heat shock protein 90 [Zea mays] | 2.53 | defense response |
| m1s0-396.5 | 19KD zein | 2.57 | nutrition reservoir |

Supplemental Table S2.2, Continued

| Band ID | Identity/Top BLAST hit | Relative expression* | GO Biological Process |
|---|---|---|---|
| u0e1-209.6 | 27kD γ-zein | 2.7 | nutrition reservoir |
| y0i0-433.7 | Alanine--glyoxylate aminotransferase 2 [Zea mays] | 2.72 | amino acid metabolism |
| h0a0-346.2 | Glutathione S-transferase GST 31 [Zea mays] | 2.79 | signal transduction |
| f0i0-156.8 | Unknown | 2.85 | Unknown |
| m1l0-273.4 | PIN domain-containing MEE21 protein [Arabidopsis thaliana] | 2.9 | embryo development |
| i0u0-440.7 | 22kD zein | 2.95 | nutrition reservoir |
| t0w0-138.0 | Zein-alpha 19C2 precursor [Zea mays] | 2.96 | nutrition reservoir |
| d0g0-177.0 | 19kD B zein | 3.11 | nutrition reservoir |
| d0g0-191.0 | 19kD D zein | 3.13 | nutrition reservoir |
| n0s0-191.7 | Zea mays protein b-32 | 3.27 | defense response |
| u0v0-169.7 | Aquaporin TIP3.1 [Zea mays] | 3.42 | Transport |
| y0i0-281.4 | Plastidic phosphate translocator-like protein1 [Zea mays] | 3.46 | Transport |
| i0n0-434.4 | Arabinogalactan protein [Zea mays] | 3.49 | Cell wall |
| f0i0-309.9 | Protein FAR-RED IMPAIRED RESPONSE 1 [Arabidopsis thaliana | 3.58 | signal transduction |
| h0a0-79.7 | Plastid phosphate/phosphoenolpyruvate translocator1 [Zea mays] | 3.72 | Transport |
| u0e1-45.3 | Flower-specific gamma-thionin [Zea mays] | 3.75 | defense response |
| n0s0-298.4 | Unknown | 3.79 | Unknown |
| i0q0-229.3 | Flower-specific gamma-thionin [Zea mays] | 3.85 | defense response |
| y0i0-156.3 | 22kD α-zein4 | 3.99 | nutrition reservoir |
| f0i0-79.9 | YT521-B-like family protein, expressed [Oryza sativa Japonica Group] | 4.07 | signal transduction |

Supplemental Table S2.2, Continued

| Band ID | Identity/Top BLAST hit | Relative expression* | GO Biological Process |
|---|---|---|---|
| m1e1-299.9 | 22kD zein | 4.42 | nutrition reservoir |
| s0w0-316.7 | 22kD zein | 4.95 | nutrition reservoir |
| u0g1-56.3 | 18kD δ-zein | 5.05 | nutrition reservoir |
| m0v0-207.8 | AAA-type ATPase family protein [Arabidopsis lyrata subsp. lyrata] | 5.27 | energy metabolism |
| m1n0-181.0 | 22kD zein | 5.4 | nutrition reservoir |
| l0m0-392.4 | Opie2 pol protein [Zea mays] | 5.5 | DNA integration |
| n0s0-287.1 | Stem-specific protein TSJT1 [Zea mays] | 5.61 | protein turnover |
| h0a0-42.1 | Oleosin Zm-I [Zea mays] (Oleosin 16kda) | 5.7 | nutrition reservoir |
| m1e1-372.6 | 19KD zein | 6.86 | nutrition reservoir |
| l0n0-375.0 | Beta-glucosidase aggregating factor [Zea mays] | 7.64 | defense response |
| u0f0-432.4 | 22kD zein | 8.37 | nutrition reservoir |
| r0k0-386.9 | Subtilisin-chymotrypsin inhibitor CI-1B [Zea mays] | 9.4 | defense response |
| s0w0-394.9 | 22kD zein | 9.56 | nutrition reservoir |
| m1w0-272.1 | 19KD zein | 10.37 | nutrition reservoir |
| u0e1-433.0 | Zea mays protein b-32 | 10.67 | defense response |
| u0g1-426.3 | 22kD zein | 17.46 | nutrition reservoir |
| b0w0-179.8 | 22kD zein | 20.48 | nutrition reservoir |
| m1s0-153.1 | 22kD zein | 25.74 | nutrition reservoir |
| s0w0-226.0 | 22KD zein | 30.02 | nutrition reservoir |
| m1w0-350.7 | 22kD zein | 65.11 | nutrition reservoir |
| u0e1-68.2 | 22kD zein | 70.61 | nutrition reservoir |
| m1e1-226.8 | 22kD zein | 81.88 | nutrition reservoir |
| m1a0-88.3 | Tryptophan aminotransferase (TA1) [zea mays] | 100 | amino acid metabolism |




Supplemental Figure S2.1: Correlation analysis of qRT-PCR and transcript profiling gene expression values. To examine reproducibility for measurement of gene expression, the values for genes confirmed by qRT-PCR were plotted against the values measured by GeneCalling transcript profiling. A Pearson correlation analysis was performed ($r = 0.80$) and the statistical significance of the linear regression was tested by ANOVA ($p < 0.001$). The value of b-32 from qRT-PCR was determined as a significant outlier by Grubbs' test and therefore the fold-change values of b-32 from both tests were removed from the plot and regression analysis.

# CHAPTER THREE

## Maize GCN2 Phosphorylates Eukaryotic Translation Initiation Factor 2α Under Amino Acid Starvation and Regulates the Endosperm Specific Transcription Factor Opaque2


*Abstract*

General control non-derepressible-2 (GCN2) plays an important role in cellular responses to amino acid availability as a regulatory protein kinase. It phosphorylates the α subunit of the trimeric eukaryotic translation initiation factor-2 (eIF2), which in turn decreases the general rate of protein synthesis in response to amino acid starvation. The phosphorylation of eIF2α enhances the translation of the transcription factor GCN4 by overcoming the inhibitory effect of the GCN4 upstream open reading frames (uORFs), resulting in increased expression of over 30 amino acid synthesis genes. Although the GCN2-like kinases are highly conserved among eukaryotes, there are no candidates of plant GCN4 homologues identified. *Mutator* tagged *GCN2* null mutants were used to characterize the GCN2 homologue in maize (*Zea mays*). ZmGCN2 shared sequence identity in the conserved domains with other GCN2 homologues. An increase of eIF2α phosphorylation in response to herbicide treatment that inhibited amino acid biosynthesis was only detected in wild type maize endosperms, not mutant, indicating that it was GCN2-dependent. Opaque2 (O2) was reported to have sequence and function similarity with GCN4, and its protein accumulation increased during induced endosperm amino acid starvation, but *O2* transcript level was unchanged. This suggested that O2 was post-transcriptionally regulated through the GCN2 kinase pathway and that O2 could be the maize GCN4 ortholog.

## *Introduction*

As a major food source, maize is deficient in essential amino acids such as lysine and tryptophan, and thus cannot be fed alone to monogastric animals due to its nutrition imbalance, limiting its value (Gibbon and Larkins, 2005b). Efforts have been made to create maize lines with better nutritional quality, and *opaque2* (*o2*) mutant was identified to have substantially increased lysine content and reduced storage protein accumulation (Mertz et al., 1964). The *O2* gene encodes an endosperm-specific b-zip transcription factor (Schmidt et al., 1990) that has pleiotropic effects on many genes, and when mutated the amino acid balance of the seed is significantly increased (Mertz et al., 1964). Transcript profiling studies have shown that O2 regulates the expression of major storage proteins, especially the 22 kDa α-zein, starch biosynthesis enzymes, pathogen defense genes and, notably, amino acid biosynthesis enzymes (Hartings et al., 2011; Hunter et al., 2002; Jia et al., 2013).

Sequence analysis of the *O2* transcript indicates that it has a 258bp 5' untranslated region (UTR), which contains three upstream open reading frames (uORFs; Lohmer et al., 1993; Schmidt et al., 1990). Previous studies found that during translation initiation, the ribosomes preferably bind to the start codon of a uORF first if it is encountered by the ribosomes scanning the 5' UTR. It reduces translation initiation of the downstream major ORF (mORF), because the dissociation of ribosomes at the stop codon of a uORF and reinitiation of ribosomes at another start site is usually inefficient (Kozak, 1984b). The uORFs of the *O2* mRNA were found to negatively affect the translation of O2, because mutations of *O2* transcripts such as lacking the 5' UTR or having all three uORFs' start codons mutated could greatly increase O2 protein levels using a heterologous tobacco

reporter system *in vivo* (Lohmer et al., 1993). There are several other transcription factors and regulatory proteins that also have uORFs in their mRNA sequences, and O2 was found to have sequence similarity with the yeast general control non-derepressible 4 (GCN4; Schmidt et al., 1990). GCN4 is also a transcription factor that belongs to the b-zip family and it was reported to be regulated post-transcriptionally through its uORFs (Hinnebusch, 1988). Additionally, O2 could partially complement the yeast *gcn4* mutant by activating the *HIS3* gene using its GCN4 binding site (Mauri et al., 1993). These findings suggest that O2 may be the maize homologue of GCN4.

The translation of GCN4 is induced by the activation of general control non-derepressible-2 (GCN2) to adapt to amino acid starvation. GCN2, first discovered as a key regulatory protein kinase in yeast, is well conserved in eukaryotes (Harding et al., 2000a; Hinnebusch, 2005; Lageix et al., 2008; Zhang et al., 2008). In yeast, amino acid starvation causes an increase of uncharged tRNA, which activates GCN2 through interaction with its C-terminal anticodon binding domain and causes a conformational change and autophosphorylation of GCN2 at threorine-882 and threorine-887 of the kinase domain (Romano et al., 1998). Activated GCN2 phosphorylates the alpha subunit of the trimeric eukaryotic initiation factor 2 (eIF2α) at serine-52. eIF2 is an important translation initiation factor and can bind to either guanosine diphosphate (GDP) or guanosine triphosphate (GTP). When bound to GTP, it is able to bind Met-tRNA to the 40S ribosomal subunit and initiate translation at the start codon of the first uORF of *GCN4* transcript. The attachment of the (eIF2.GTP.Met-tRNA) ternary complex (TC) to the 40S subunit causes translation initiation and GTP to be hydrolysed to GDP, releasing the inactive (eIF2.GDP) complex. Phosphorylation of eIF2α inhibits the exchange of eIF2-GDP to eIF2-GTP, thus decreasing

the concentration of TC and the rate of translation initiation as well as global protein synthesis (Kimball, 1999). After the ribosomes dissociate at the first uORF stop codon, approximately 50% of the 40S subunits remain attached to the mRNA and continue to scan. Under normal conditions, the rescanning 40S subunits quickly recognize the next start codon, rebind TC, reinitiate translation at the last uORF and dissociate again at the stop codon, causing the translation of mORF of *GCN4* to be skipped. During amino acid starvation, the decreased concentration of TC slows down the reinitiation of translation by causing part of the rescanning 40S ribosomes to rebind the TC until they scan past the last uORF, and reinitiate at the *GCN4* mORF instead. Therefore, it enhances the translation of GCN4 by overcoming the inhibitory effect of the *GCN4* uORFs, resulting in increased expression of over 30 amino acid synthesis genes, as well as genes involved in a wide range of other cellular processes (Hinnebusch, 1988, 2005; Natarajan et al., 2001). Therefore, the uORFs of *GCN4* form a *cis* regulatory element that allows translation of *GCN4* mORF only when amino acid availability is deprived. This regulation depends on the presence and spacing of the uORFs in its 5' UTR (Hinnebusch, 2005). The regulation of GCN4 translation by GCN2 is part of the general amino acid control (GAAC) response, a key pathway for yeast cells to maintain homoeostasis and survive conditions of amino acid starvation. Although O2 and GCN4 shared structural and functional similarity and O2 was found to be regulated in a similar way as GCN4, it is not clear whether this regulation of O2 is part of the GAAC in maize.

The GCN2-like kinases are highly conserved among eukaryotes. The phosphorylation site at serine-52 by GCN2 is also highly conserved in eIF2α homologues (Zhang et al., 2008). In all plant species with full genome data available, GCN2 appears to

be a single copy and appears to be the only eIF2a kinase (Halford, 2006). In Arabidopsis, AtGCN2 is shown to be activated and phosphorylate AteIF2α in response to herbicide-induced amino acid starvation (Lageix et al., 2008). *Atgcn2* null mutants that grow normally under standard conditions are more sensitive compared to wild type plants when they are treated with amino acid biosynthesis inhibiting herbicides, such as glyphosate and chlorosulfuron (Zhang et al., 2008). AtGCN2 is also activated during UV exposure, cold shock, wounding, and exposure to methyl jasmonate, 1-aminocyclopropane-1-carboxylic acid (ACC) and salicylic acids, and the activation of AtGCN2 is linked to a reduction in global protein synthesis (Lageix et al., 2008). These findings suggest that GCN2 is essential for plant growth in abiotic stress conditions and could play a role in plant defense responses to insect herbivores. In wheat (*Triticum aestivum*), overexpression of TaGCN2 causes a significant decrease of total free amino acid concentration in the grain, indicating that TaGCN2 plays an important role in the regulation of genes encoding enzymes of amino acid biosynthesis in wheat. It is also reported that TaGCN2 is involved in sulfur signaling (Byrne et al., 2012). Although these studies have shown that the GAAC system exists in plants, at least in part, there are no obvious candidates of GCN4 homologues identified in Arabidopsis or wheat (Halford, 2006).

In this study, *Mutator* tagged GCN2 null mutants are used to characterize the GCN2 homologue in *Zea mays*. The role of ZmGCN2 and its translational regulation of O2 during amino acid starvation is investigated. We found that phosphorylation of ZmeIF2α by ZmGCN2 occurs during herbicide induced amino acid starvation, which causes an increased translation of the maize b-zip transcription factor O2, suggesting O2 as a homologue of GCN4, in the maize endosperm. This study demonstrates that the GAAC

system is conserved in maize endosperm and O2 could be a maize GCN4 homologue. These findings could provide insights into the link between amino acid signaling, stress sensing and regulating, crop yield and quality.

## *Materials and Methods*

### *Plant Material and Growth Conditions*

Mutant alleles *gcn2-1*, *gcn2-2* and *gcn2-3* were *Mu*-tagged maize *GCN2* alleles from the TUSC population developed by Pioneer Hi-Bred, and mutant alleles *gcn2-4*, *gcn2-5* and *gcn2-6* were from the UniformMu population developed by University of Florida (Acc. Nos: UFMu00599, UFMu01828 and UFMu03483, respectively) (McCarty et al., 2005). Allele *gcn2-1* was backcrossed to the maize (*Zea mays L.*) inbred line B73 for six generations to create a nearly-isogenic genomic background, whereas alleles *gcn2-4*, *-5* and *-6* share the same genetic background of W22 inbred line (McCarty et al., 2005). Allele *gcn2-3* was not backcrossed to an inbred line sufficiently but it was used to demonstrate that *Mu* insertion in the ZmGCN2 intron did not completely abolish ZmGCN2 activity. B73+, B73 *gcn2-1*, *gcn2-3* wild type and *gcn2-3* mutant plants were grown at Elm Mott, TX in the summer of 2012 and 2013. W22+ and W22 *gcn2-5* plants were grown in soil in a growth room at 26°C with a 16/8 h day/night photoperiod.

For *Mu* insertion genotyping, *ZmGCN2* transcript cloning, quantitative real-time polymerase chain reaction (qRT-PCR) and western blotting, B73+, B73 *gcn2-1*, *gcn2-3* wild type, *gcn2-3*, W22+ and W22 *gcn2-5* kernels at 18 days after pollination (DAP) were harvested and kept frozen at -80°C. For allele *gcn2-1*, a developmental series of 10, 14, 22 DAP kernels were also harvested and kept frozen. Three ears of each genotype were used

as three biological replicates. Six endosperms or embryos of each ear were dissected and ground to a fine powder in liquid nitrogen. For tissue specific gene expression analysis, roots and the third leaves of 14-days-old seedlings from both wild type and mutant plants were harvested and kept frozen. Tissues from 3 seedlings were combined as one biological replicate.

*Genotyping of Mu Insertion Alleles*

Genomic DNA was extracted from young maize leaves and developing kernels with cetyltrimethylammonium bromide (CTAB) extraction buffer (100mM Tris-HCl, pH 7.5, 700mM NaCl, 50mM EDTA, pH 8.0, 1% CTAB (w/v) and 140mM β-mercaptoethanol) (Gao et al., 2008). Briefly, fresh or frozen tissues were homogenized with 400 µl of CTAB buffer and incubated at 65°C for 1 hr. Four-hundred µl of 24:1 chloroform:isoamyl alcohol was then added to each extraction and mixed thoroughly. After centrifugation the aqueous phase was transferred to fresh tubes and genomic DNA was precipitated with 70% ethanol. DNA was resuspended in 50 µl DI water after a brief air-dry.

PCR genotyping was performed using combinations of a *Mu* terminal inverted repeat (*Mu*-TIR) specific primer and gene-specific primers that flank the approximate insertion sites provided by the resources (primer sequences listed in Supplemental Table S3.1). The PCR program was as follows: 95°C initial denaturing for 2 min, 40 cycles of 95°C denaturing for 15 s, 58-60°C annealing for 15 s and 72°C extension for 1 min, followed by a final extension for 2 min at 72°C. The genotypes were determined by the presence or absence of PCR products from primer combinations. The exact insertion sites were confirmed by sequencing the PCR products of each primer combination.

*ZmGCN2 Transcript Sequencing*

Primers were designed to span the entire transcript according to the gene model (Acc. No. GRMZM2G052142) from Gramene (http://ensembl.gramene.org/Zea_mays/Info/Index). Two pairs of primers were designed based on the sequence directly upstream of the *ZmGCN2* start codon from the genomic sequence available in order to sequence 5' UTR because of the lack of predicted splicing sites. Total RNA was isolated from 18 DAP endosperm using Purelink™ Plant RNA Reagent (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. First-strand cDNA was synthesized from 1 μg of total RNA with qScript™ cDNA SuperMix (Quanta Biosciences, Gaithersburg, MD) and used as the template for subsequent PCR reactions to sequence *ZmGCN2* transcript in both wild type and mutant plants. PCR was performed with Phusion® High-Fidelity DNA polymerase (New England Biolabs, Ipswich, MA). PCR products were purified from the agarose gel using illustra™ GFX™ PCR DNA and gel band purification kit (GE Healthcare, Piscataway, NJ) and were either directly sequenced or cloned into pGEM®-T Easy Vector systems (Promega, Madison, WI) followed by sequencing. Sequences were assembled and the consensus sequence was translated to amino acid sequence followed by multiple sequence alignment in Geneious (Biomatters, Auckland, New Zealand). Multiple alignment was made using the ClustalW alignment algorithm in Geneious with a gap open cost of 10 and gap extend cost of 0.1.

The RNA-Seq data used to confirm the sequenced 5' UTR were obtained from NCBI Sequence Read Archive (http://www.ncbi.nlm.nih.gov/sra; Acc. No. SRX313720). Reads were aligned to Zea mays B73 genome assembly AGPv3 using TopHat2 (Kim et

al., 2013) and visualized using Integrative Genomics Viewer 2.3 (Thorvaldsdottir et al., 2013).

*3' Rapid Amplification of cDNA Ends (RACE)*

The 3' end of the *ZmGCN2* transcript was amplified by RACE using an ExactSTART™ Eukaryotic mRNA 5'- & 3'- RACE Kit (Epicentre, Madison, WI) and a ImProm-II™ Reverse Transcription System (Promega, Madison, WI), following the procedure described below.

A mixture of 1 µg of total RNA and 1 µl cDNA synthesis primer (5' -CGAGCA CAGAATTAATACGACTCACTATAGGT12VN) from the RACE kit was incubated at 70°C for 5 min and immediately chilled on ice for 5 min. First-strand cDNA was synthesized by adding the appropriate buffer and MMLV reverse transcriptase from Promega into the RNA and primer mix, and then incubating at 25°C for 5 min, 42°C for 1 hr and 70°C for 15 min. Outer 3' RACE PCR was performed using a forward primer (ZmGCN2_8F) and a reverse primer that specifically identifies the adaptor in the cDNA synthesis primer (5' TAGACTTAGAAATTAATACGACTCACTATAGGCGCGCCAC CG). A nested PCR was performed using primers ZmGCN2_9F, 10F with ZmGCN2_8R (Supplemental Table S3.1).

*Cob Culture Treatment*

Cob culture was performed according to Cheng and Chourey with minor modifications (Cheng and Chourey, 1999). The cob culture medium consisted of Chu's N6 salt (PhytoTechnology Laboratories, Shawnee Mission, KS) with 15g/L sucrose and 4g/L Gellan Gum powder (PhytoTechnology Laboratories, Shawnee Mission, KS). The

herbicide chlorosulfuron (Sigma, St. Louis, MO) was added to the medium prior to solidification to a final concentration of 20 p.p.b. for the treatment group. Maize developing ears (B73+ and *gcn2-1*) were harvested at 18 DAP and sectioned into blocks with 2 rows, 6 kernels attached to one end, and the vascular system attached to the other end. To make pairwise comparison, blocks from the same ear were sectioned and placed in both control and treatment media. Three ears were used for each treatment and multiple blocks were sectioned from each ear. After sectioning, the blocks were incubated with the vascular system inserted into the cob culture medium at 30°C for 16 hrs in the dark. The kernels were immediately frozen in liquid nitrogen after incubation and stored at -70°C for further use.

*ZmGCN2 Gene Expression Analysis*

First-strand cDNA was prepared the same way as *ZmGCN2* transcript sequencing, followed by a 10-fold dilution in water. qRT-PCR was done with PerfeCTa® SYBR® Green FastMix® (Quanta Biosciences, Gaithersburg, MD) using a 72-well rotor in the Corbett Rotor-Gene™ 3000 (Qiagen, Velancia, CA). The PCR was carried out as: 50ºC hold for 2 min for auto gain optimization, 95ºC initial denaturing for 10 min, 50 cycles of 95ºC for 15 s and 60ºC for 1 min. Melting curves were obtained by heating from 55˚C to 95˚C with a 1˚C per second ramp rate to confirm single amplicons. Expression levels of genes were normalized against the expression of RRB1 (Sabelli et al., 2005) based on the preliminary data where RRB1 showed expression stability. The normalization of gene expression was calculated with the Q-Gene Core Module file (Simon, 2003). Statistical differences of gene expression levels between wild type and mutant plant tissues were

evaluated with unpaired two-tailed student's t-test using the JMP statistical software (SAS Institute Inc., Cary, NC).

Primers were designed using Primer3 Plus (www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) and are listed in Supplemental Table S3.1. They were designed to span exon-exon junctions in order to control for genomic DNA contamination.

*Kernel Protein Extraction, SDS-PAGE and Western Blotting*

Total soluble proteins from dissected maize endosperms were extracted with borate extraction buffer [12.5 mM NaBO3, 1% (w/v) sodium dodecyl sulfate and 2% (v/v) 2-mercaptoethanol; (Lending et al., 1989). Borate extraction buffer was added to weighed ground endosperms to a 20:1 ratio and incubated with shaking for at least 2 h at RT. Insoluble cell debris was removed from the crude extract by centrifugation for 15 min at 16,000×g. The cleared protein extracts were either used for SDS-PAGE or zein protein fractionation.

Three hundred µL of total protein extracts were mixed with 700 µL 100% ethanol to precipitate non-zein proteins at -20°C overnight. After centrifuge, the supernatant which contained the zein fractions were completely dried using a Vacufuge® vacuum concentrator (Eppendorf, Hauppauge, NY) and resuspended in 200 µL DI water. The pellets which contained non-zein fractions were washed twice with 70% ethanol and resuspended in 200 µL 1X protein sample buffer (0.04M Tris-HCl, pH 6.8, 2% (w/v) SDS, 0.1M DTT, 10% (v/v) glycerol and 0.02% (w/v) bromophenol blue).

Twenty-five µg of total protein or equal volume of fractioned protein from each sample was separated by 12.5% SDS-PAGE in 1X SDS-PAGE running buffer (25mM Tris, 192mM glycine, 0.1% (w/v) SDS) and then transferred to a Protran nitrocellulose

membrane (Whatman, Pensacola, FL) using a TE 22 Mighty Small Transphor Tank Transfer Unit (GE Healthcare, Piscataway, NJ). The quality of protein transfer was visually checked by staining the membrane with Ponceau S (0.1% (w/v) in 5% (v/v) acetic acid). The membrane was blocked with 3% non-fat dry milk powder in 1X TBS buffer (50mM Tris-HCl, Ph 7.5, 200mM NaCl) for 1 h at room temperature or at 4°C overnight with shaking.

Polyclonal antibodies against ZmGCN2 were generated in rabbits using the affinity purified peptide of 6X His-tagged ZmGCN2 RWD domain as antigens. The RWD domain antigenic region is at the N-terminus of ZmGCN2 and the amino acid sequence of the peptide is RKAAKDHAAQLEGDETALDEELTALASILGEDFKVTSESPHTRCNIC IRPYSDDMGFEDLNISAILIVICFPGYPHKCPKLRILPEKNLSKEDTDRLLSLLIDQA NIYSREGRVMIFNLFEAAQEFLSEIAPARVSVSTASFLGLSSTTDDNVEVGFDSDP YL. Antibodies to detect eIF2α and O2 were provided by Karen Browning and Angelo Viotti, respectively. The anti-phospho-eIF2α antibody was commercially available (Cat. No. ab32157, Abcam, Cambridge, MA), and it was raised against a well conserved region surrounding phospho-Ser51 of human eIF2α. Membranes were incubated with primary antibodies diluted in TBST (10mM Tris-HCl, pH 8.0, 150mM NaCl, 0.1% (v/v) Tween-20; dilution 1:1000 to 1:3000, based on the antibody titer) for 1 h at room temperature or 4 ºC overnight, washed with TBST and then incubated for 1 h at room temperature with secondary antibodies (horseradish peroxidase-conjugated goat anti-rabbit; Invitrogen, Carlsbad, CA) diluted in TBST (1:30,000). Membranes were then treated with 1ml Amersham ECL Prime (GE Healthcare, Piscataway, NJ) for 2 min and the signals were detected using ImageQuant LAS 4000 imaging system (GE Healthcare, Piscataway, NJ).

The intensity of bands was quantified using the ImageJ software (Schneider et al., 2012) and statistical differences of protein expression levels between different treatment and genotypes were evaluated with unpaired two-tailed student's t-test with the JMP statistical software (SAS Institute Inc., Cary, NC).

## *Results*

### *Identification of Mutator Induced Zmgcn2 Mutants*

The gene model of maize GCN2 (ZmGCN2) was available from maizesequence.org (Acc. No. GRMZM2G052142). A BLAST search using the predicted ZmGCN2 protein sequence against the maize B73 genome indicated that there was a single copy of ZmGCN2 on maize chromosome 2. Six *Mutator* (*Mu*)-tagged *gcn2* mutant alleles were obtained and the loci of the *Mu* insertions were identified. Alleles *gcn2-1*, *gcn2-2*, *gcn2-3* were obtained from the TUSC population developed by Dupont Pioneer, and alleles *gcn2-4*, *gcn2-5*, *gcn2-6* were from the UniformMu population developed by University of Florida (Settles et al, 2007; Stock Nos. UFMu00599, UFMu01828 and UFMu03483, respectively). Genotyping was done with combinations of primers flanking the insertions and a primer directed against the *Mu* terminal inverted repeat. These PCR products were purified and sequenced, and the positions of the *Mu* insertions are shown in Figure 3.1. Mutant allele *gcn2-1* has a *Mu* insertion in the start codon of the first exon, disrupting the *ZmGCN2* open reading frame. Insertions of *gcn2-2*, *gcn2-4*, *gcn2-5* are found 5' upstream of the transcript start codon (-45, -41, and -24, respectively). Insertions *gcn2-3* and *gcn2-6* are within the first intron. Allele *gcn2-1* has been back-crossed into the maize inbred line B73 for six generations, and *gcn2-4*, *gcn2-5* and *gcn2-6* are nearly-isogenic to the maize

inbred line W22. Allele *gcn2-1* was used as the main material for this study, with *gcn2-5* as a second allele to confirm the findings.

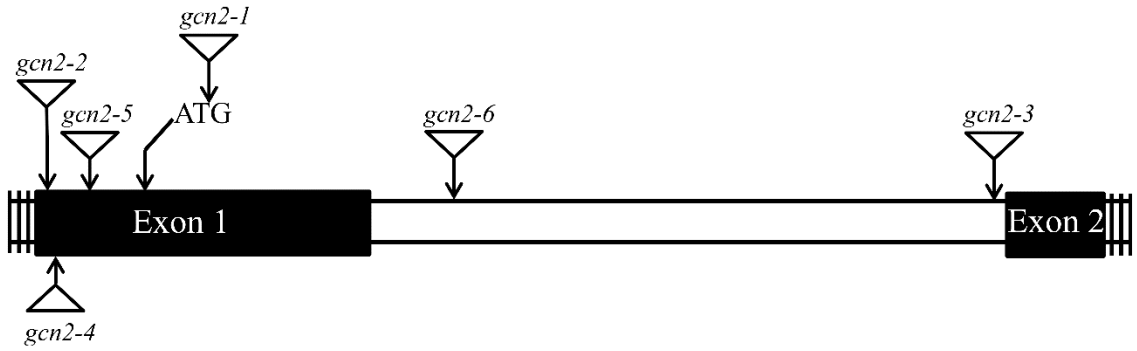



Figure 3.1: Relative insertion sites of *Mutator* transposable element within the GCN2 gene. Alleles were obtained from Dupont Pioneer TUSC and the Uniform Mu population. Allele *gcn2-1* contains a *Mu* insertion in the first exon of the gene, inserting into the start codon. Alleles *gcn2-2*, *gcn2-4*, and *gcn2-5* contain insertions in the 5' UTR region, whereas alleles *gcn2-3* and *gcn2-6* contain insertions in the first intron. Exon 1 is indicated by the black box.

*ZmGCN2 Transcript Structure*

To sequence the entire *ZmGCN2* transcript, primers were designed to span the coding region, and up to 200 bp upstream of the start codon and 300 bp downstream of the stop codon, based on the splicing sites predicted by the gene model. cDNA was synthesized using total mRNA extracted from both wild type and mutant developing endosperms and was used as the PCR template. Genomic DNA was used as a control for genomic DNA contamination as well as confirmation of predicted splicing sites. PCR products were sequenced and assembled in Geneious (Version R7 developed by Biomatters Ltd). To confirm the sequenced nucleotides upstream of the start codon are indeed part of the 5' UTR, previously published RNA-Seq data were obtained from NCBI Sequence Read

Archive (http://www.ncbi.nlm.nih.gov/sra), aligned to *Zea mays* B73 genome assembly (version: AGPv3) and visualized with Integrative Genomics Viewer 2.3 (Acc. No. SRX313720; Oliveira-Garcia and Deising, 2013; Thorvaldsdottir et al., 2013). At least 30 reads were aligned to the region surrounding the *ZmGCN2* start codon (covering both upstream and downstream sequences), confirming the sequenced 5' UTR (not shown).

The 3' end of *ZmGCN2* was amplified by rapid amplification of cDNA ends (RACE), resulting in the amplification of two PCR products (Supplemental Figure S3.1). The products were 854 bp and 870 bp in length. Two polyadenylation sites were identified, 554bp and 570bp downstream of the stop codon, respectively. The nucleotide sequences of these two products were identical and matched the genomic sequence perfectly, despite the differences in length. The sequences were submitted to Genbank. The *ZmGCN2* transcript has an open reading frame of 3753 bp and alignment of the sequenced transcript to the corresponding genomic region (chromosome 2: 34393536-34432082) indicates that there are 30 exons in the transcript.

*ZmGCN2 Protein Structure*

The *ZmGCN2* transcript encodes a protein of 1250 amino acid residues, with a predicted molecular weight of 141.13 kDa. The ZmGCN2 protein has the predicted functional domains that are consistent with the homologues in other eukaryotes: an N terminus RING-finger, WD40, DEAD-box helicase (RWD) domain, an eIF2α kinase domain, a histidyl-tRNA synthetase-like regulatory domain and a C-terminal anticodon binding domain (Figure 3.2A). Multiple alignment of GCN2 protein sequences from various organisms showed that ZmGCN2 shared 80% amino acid sequence identity with wheat (*Triticum aestivum*), 49% identity with Arabidopsis (*Arabidopsis thaliana*), and less

than 25% identity with yeast (*Saccharomyces cerevisiae*) (Figure 3.2B). Although ZmGCN2 has a much higher sequence similarity with GCN2 homologues from cereal plants, the main functional domains are well conserved among the compared species, indicating that ZmGCN2 could function similarly as its homologues.

*ZmGCN2 Transcript Expression Analysis*

In Arabidopsis and wheat, the *GCN2* transcript has been shown to be expressed in all tissues, with no significant differences among tissues and developmental stages (Byrne et al., 2012). The *ZmGCN2* transcript expression profile from the maize gene expression atlas (Sekhon et al 2011; http://www.maizegdb.org) suggests similar findings, except that *ZmGCN2* transcript level increased during endosperm development. Quantitative real-time PCR (qRT-PCR) was performed to analyze the expression of *ZmGCN2* in maize root, leaf, developing embryos and endosperms from both wild type and mutant plants. qRT-PCR showed that *ZmgGCN2* transcript was still being transcribed in *gcn2-1* homozygous mutant tissues, and the expression level was significantly reduced in mutant root and endosperm tissues, but was similar to the wild type in mutant leaves and embryos (Figure 3.3A). The expression of *ZmGCN2* increased during endosperm development, with a significant increase from 10 DAP to 14 DAP, peaking at 18 DAP and decreased slightly at 22 DAP, the trend of which could also be seen in *gcn2-1* mutant endosperms, although at a much lower expression level (Figure 3.3B). *ZmGCN2* expression was also found to be significantly reduced in allele *gcn2-5* 18 DAP endosperms (Supplemental Figure S3.2). The residual transcription of mutant *gcn2* mRNA could be explained by the *Mu* insertions at the first exon of the transcripts that do not disrupt the process of *Mu*-containing transcription but affect transcript stability. This

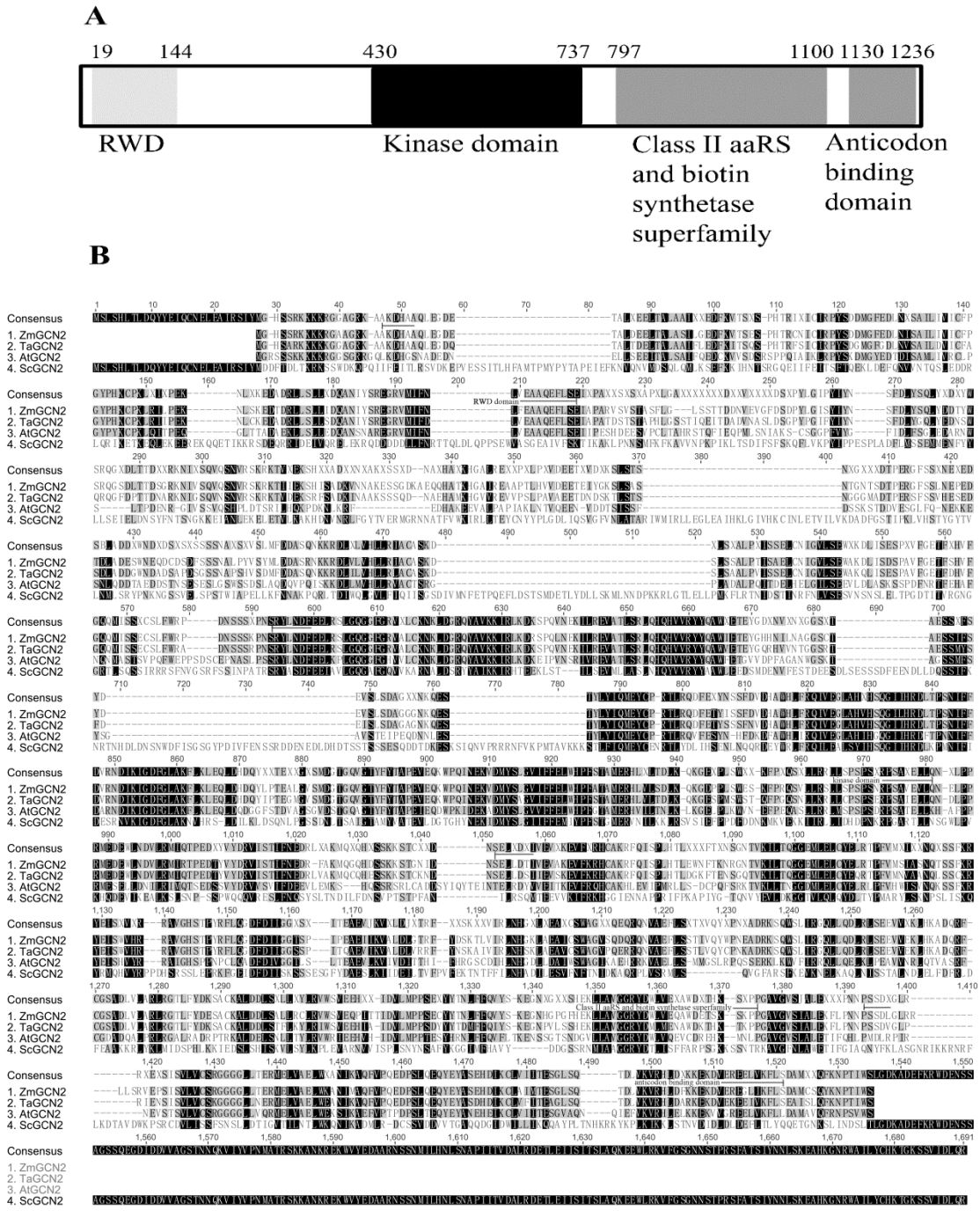



Figure 3.2: Analysis of the structure of ZmGCN2. A) Schematic diagram of ZmGCN2 protein functional domains. B) Multiple alignment of GCN2 protein sequences. The main functional domains are well conserved among species, indicating that ZmGCN2 could function similarly as the characterized GCN2 protein in other species. Identical amino acids at the same position are highlighted in black, and lighter highlights indicate less similarity. GCN2 protein sequences are obtained from NCBI. The accession numbers are: CCA41210.1 for wheat, NP_191500.2 for Arabidopsis, and NP_010569.3 for yeast.

effect could also be tissue specific (Bennetzen, 1996). Based on sequenced alleles *gcn2-1* and *gcn2-5*, the *Mu* insertions replaced sections of the *ZmGCN2*transcript with *Mutator* sequences from the site of insertion to the intron splice site (junction of exon1 and exon 2), resulting in a premature stop codon in both cases (Figure 3.4). These changes in the mutant transcripts suggested that *ZmGCN2* protein would not be produced in these mutant plants.

*ZmGCN2 Protein Accumulation is Not Detectable in Mutant Plants*

To detect ZmGCN2 protein accumulation, antiserum was raised against recombinant 6X His-tagged RWD domain, which is present in the N-terminus of ZmGCN2. Western blot analysis of total soluble protein extracts from 18 DAP

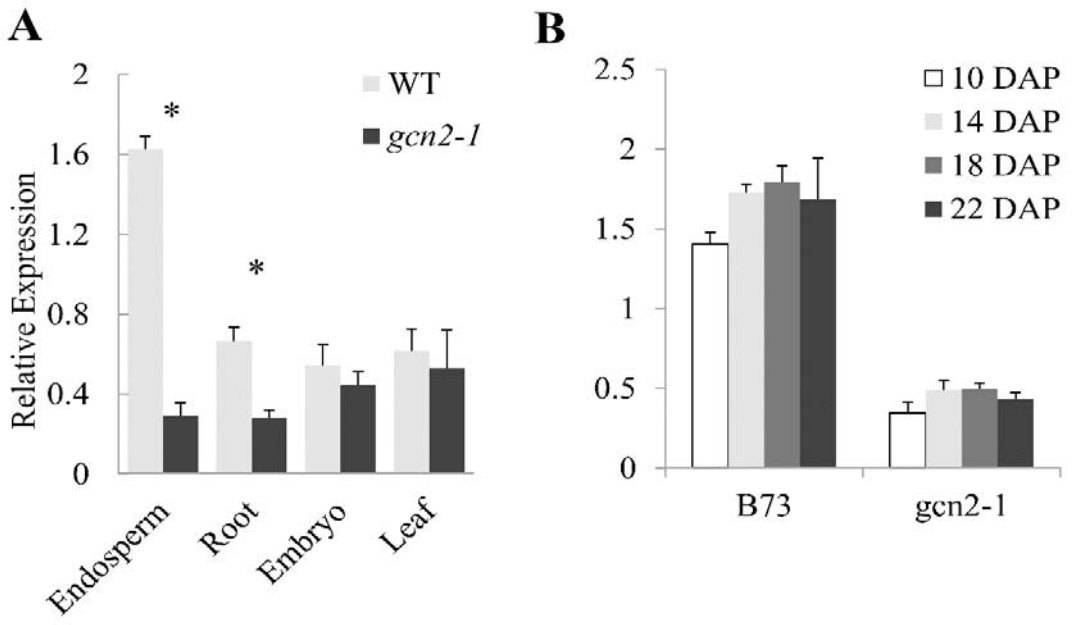



Figure 3.3: Transcript expression analysis of *ZmGCN2* in different tissues. Expression levels of the *ZmGCN2* gene were analyzed A) in different maize tissues from wild type and *gcn2-1* mutant plants. Asterisks indicate $p < 0.05$ (n=3) B) during endosperm development in B73 and *gcn2-1* mutant. *ZmGCN2* expression levels of B73 were significantly higher than that of *gcn2-1* in all four developmental stages ($p< 0.001$, n=3).

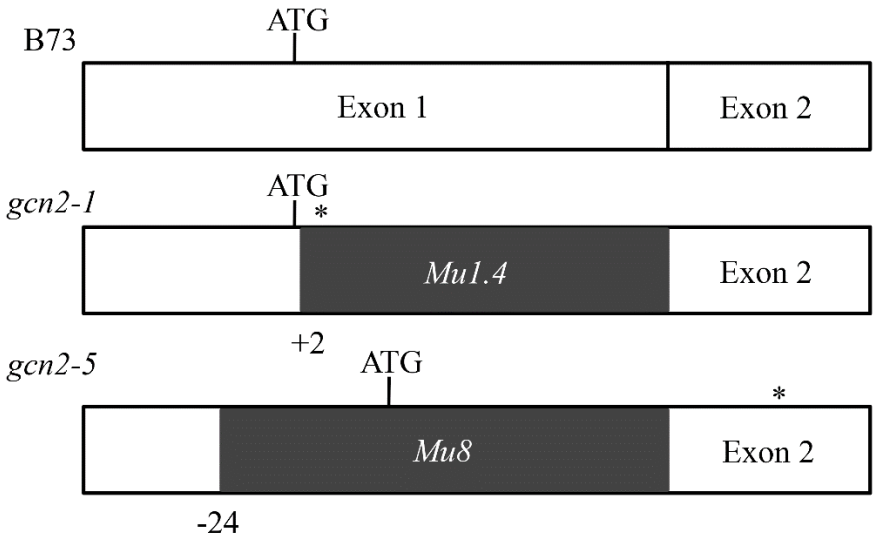



Figure 3.4: 5' end transcript structures of *ZmGCN2* and *Zmgcn2* transcripts. The white areas indicate exons; dark areas indicate replacement of exon 1 (and partial 5' UTR in *gcn2-5*) by *Mu* transposable elements. BLAST results of sequenced *Mu* insertion transcripts indicated that in *gcn2-1* the insertion was *Mu1.4*, whereas in *gcn2-5* the insertion was *Mu8*. Numbers indicate the loci of *Mu* insertions. Asterisks indicate the loci of premature stop codons. *Mu8* insertion at *gcn2-5* causes a frame shift and the premature stop codon is in exon 2.

endosperms detected a protein of ~150 kDa in the wild type endosperms but not in the *gcn2-1* or *gcn2-5* mutants. For allele *gcn2-3*, ZmGCN2 was detected in both wild type and mutant endosperms (Figure 3.5). This is consistent with the structures of the mutant nucleotide sequences, where the disruption of *ZmGCN2* mORF by *Mu* insertions resulted in a premature stop codon upstream of the RWD domain, whereas the intron insertion of *gcn2-3* did not cause any alteration of the mORF. Although *ZmGCN2* transcript was not completely knocked out in the *gcn2-1* and *gcn2-5* mutant plants, ZmGCN2 protein accumulation was not detectable.

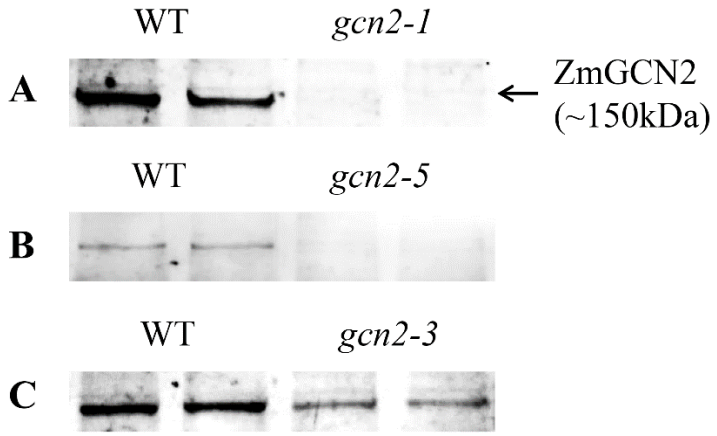



Figure 3.5: Western blot analysis of ZmGCN2 protein in 18 DAP maize endosperms. ZmGCN2 was present in wild type plants but absent in *gcn2-1* and *gcn2-5* mutant plants. ZmGCN2 was still detectable in *gcn2-3* mutant, although at a reduced level.

*ZmGCN2 is Necessary for the Phosphorylation of eIF2α in Maize*

To determine if ZmGCN2 functions similarly as the other GCN2 homologues, a modified cob culture experiment was performed using 18 DAP developing ears from both wild type and mutant plants (Cheng and Chourey, 1999). Ears were sectioned with cob tissues attached to function as the vascular system, and were placed with the vascular system inserted into the cob culture media containing 20 p.p.b. chlorosulfuron. The cultures were incubated at 30 °C for 16 hrs in the dark and then harvested and frozen in liquid nitrogen. Total soluble protein, zein proteins and non-zein fractions were extracted and fractionated from dissected endosperms, and were analyzed on SDS-PAGE gels and by western blotting. The phosphorylation site of eIF2α by GCN2 is highly conserved in eukaryotes (Supplemental Figure S3.3), which enabled the use of the commercially available anti phospho-eIF2α that recognizes the residues surrounding Ser51 of human

eIF2S1 to detect phospho-eIF2α in maize (Lageix et al., 2008). As shown in Figure 3.6A, phosphorylation of eIF2α increased significantly in chlorosulfuron treated wild type endosperms compared to control, whereas no signal of eIF2α phosphorylation could be seen in either control or treated *gcn2-1* mutant samples. However, the accumulation of total eIF2α protein was similar and remained unchanged in all tested samples. There was also signal of phosphorylation of eIF2α detected in the wild type control endosperms, although at a much lower level ($p<0.05$, $n=3$). It could be that the relatively long incubation period was not optimal for endosperm growth and that the culturing procedure induced a stress response. Constitutive phosphorylation of eIF2α is unlikely, because no p-eIF2α was detected in the endosperm tissues that were not incubated on the media but frozen upon harvest from the field (Supplemental Figure S3.4). A similar response was also seen in maize seedlings, where increased accumulation of p-eIF2α was observed only in wild type plants but not in *gcn2-1* mutants after herbicide treatment (Supplemental Figure S3.5). Similar to Arabidopsis mutants, *gcn2-1* mutant plants grew normally in soil but were more sensitive to prolonged chlorosulfuron treatment (not shown). Consistent with the above, phosphorylation of eIF2α was detected in both *gcn2-3* wild type and mutant endosperm treated with chlorosulfuron, but was not detected in *gcn2-5* mutant endosperms (Supplemental Figure S3.6). These observations suggest that ZmGCN2 is required for phosphorylation of eIF2α in response to chlorosulfuron treatment that interferes with amino acid biosynthesis and causes amino acid starvation.

*Translation of O2 is Increased during Amino Acid Starvation*

Western blots indicated that O2 protein expression increased in the herbicide treated wild type endosperms compared to the untreated tissues, but did not change in gcn2-

1 mutants (Figure 3.6A). Densitometry analysis showed accumulation of O2 was increased by 1.4 fold ($p < 0.05$, n=3) in wild type endosperms when amino acid starvation was induced by chlorosulfuron (Table 3.1). Similarly, O2 protein was increased in the treated *gcn2-3* wild type endosperm, but did not change in *gcn2-5* endosperm (Supplemental Figure S3.6). Moreover, qRT-PCR analysis showed that the transcript level of *O2* did not change during herbicide treatment (Figure 3.6B). The slight increase of *O2* after treatment in both genotypes could also be caused by the long incubation time. The increased O2 protein accumulation together with the barely affected *O2* transcript expression level in wild type endosperms strongly suggests that the accumulation of O2 protein was post transcriptionally regulated by phosphorylation of eIF2α and this regulation was GCN2-dependent.

## *Discussion*

Phosphorylation of eIF2α by GCN2 has been shown to be a highly conserved regulatory mechanism in eukaryotes (Harding et al., 2000a; Hinnebusch, 2005; Zhang et al., 2008). In yeast, phosphorylation of eIF2α decreases the general rate of protein synthesis in response to amino acid starvation, but enhances the translation of the transcription factor GCN4 by overcoming the inhibitory effect of the *GCN4* upstream open reading frames (uORFs), resulting in increased expression of over 30 amino acid synthesis genes (Hinnebusch, 2005). This GAAC system plays an important role in maintaining cell homeostasis and has been shown to be at least partially conserved in plants, with no candidates of plant GCN4 homologues identified. In this study, we have shown that the maize GCN2 homologue, ZmGCN2, phosphorylates eIF2α under amino acid starvation,

and this phosphorylation of eIF2α results in an increased protein accumulation of maize endosperm specific b-zip transcription factor O2.

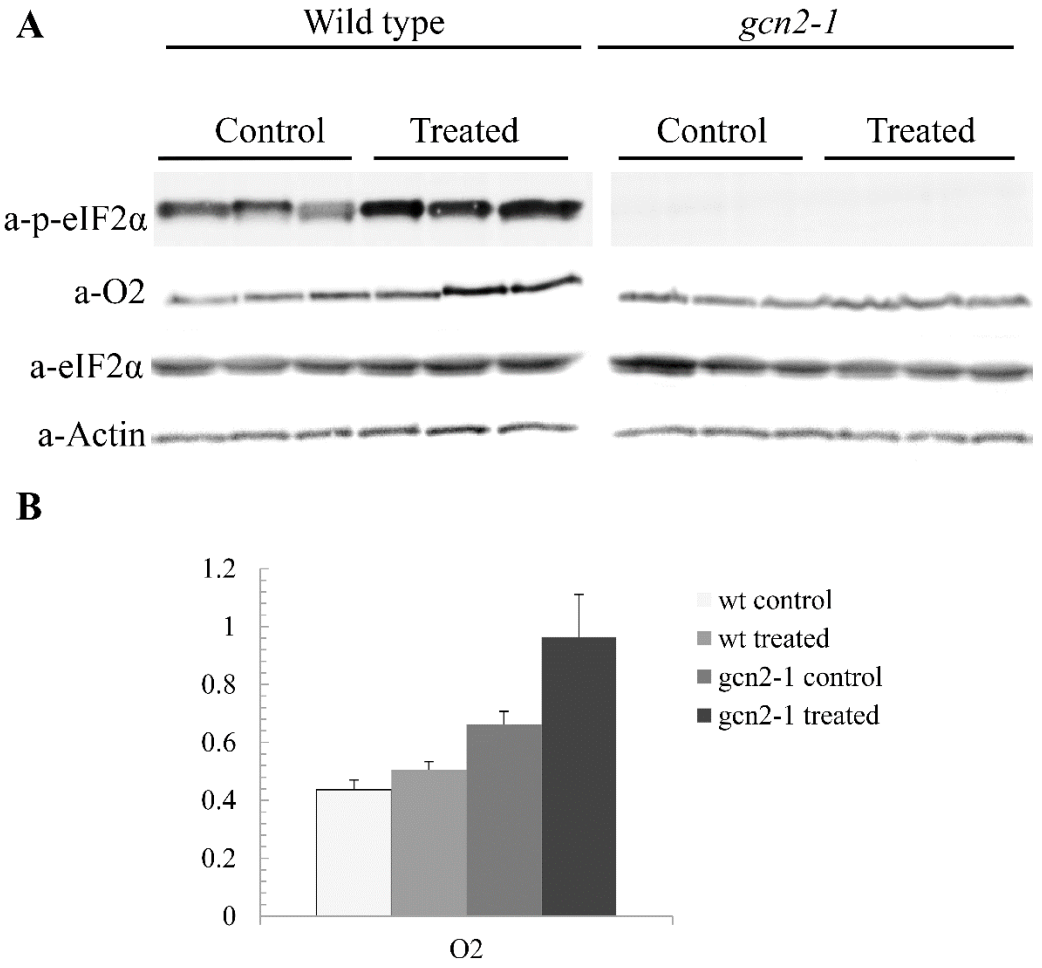



Figure 3.6: Expression analysis of herbicide treated maize developing endosperms. A) The phosphorylation level of eIF2α significantly increased after incubation (16 hr, dark, 30 °C) in media with 20 ppb chlorosulfuron. The accumulation level of O2 also significantly increased after incubation. The accumulation of non-phosphorylated eIF2α did not change. Actin is used as a loading control. B) *O2* transcript analysis after cob culture. The level of *O2* transcript did not increase after the treatment. Although there were some changes in O2 transcript levels, they were not significant, which could be caused by the long incubation of cob culture.

Table 3.1: Densitometry analysis of the western blots of herbicide treated 18 DAP maize endosperms (n=3). P-values were calculated using two tailed Student's t-test. Italicized figures are significantly different at a level of $p<0.05$ (n=3). ND: non detectable.

| | WT control | | WT treated | | | |
|---|---|---|---|---|---|---|
| Protein | Mean | SD | Mean | SD | Fold change | p-value |
| O2 | 185.11 | 8.10 | 250.84 | 25.45 | 1.40 | *0.01* |
| p-eIF2α | 127.21 | 11.50 | 195.58 | 30.57 | 1.54 | *0.02* |
| eIF2α | 192.39 | 2.13 | 201.92 | 13.11 | 1.05 | 0.28 |
| Actin | 82.35 | 3.12 | 88.25 | 6.98 | 1.07 | 0.25 |
| | *gcn2-1* control | | *gcn2-1* treated | | | |
| Protein | Mean | SD | Mean | SD | Fold change | p-value |
| O2 | 206.59 | 12.83 | 203.64 | 21.43 | 0.85 | 0.99 |
| p-eIF2α | ND | | ND | | | |
| eIF2α | 203.84 | 35.02 | 28.01 | 3.62 | 0.90 | 0.39 |
| Actin | 82.78 | 2.33 | 86.97 | 5.26 | 0.28 | 1.05 |

ZmGCN2 has similar functional domains as its characterized homologues and it is activated by herbicide induced amino acid starvation. ZmGCN2 is essential for maize growth in stress conditions. Both wild type and *gcn2* mutant seedlings grew normally in soil. However, *gcn2* mutant seedlings were more sensitive compared to the wild type seedlings after treated with herbicides that inhibited amino acid biosynthesis. Mutant leaves exhibited bleaching and senescence, which could be seen in both Arabidopsis and maize (Zhang et al., 2008).

During amino acid starvation, the increased level of uncharged tRNA activates ZmGCN2, which leads to ZmGCN2 phosphorylation of ZmeIF2α and the reduction of global translation initiation rate. Reduced translation initiation rate causes the ribosomes to reinitiate at the start codon of the *O2* mORF, thus increasing the translation of O2, but the alteration of transcript level of *O2* is minimal. It is consistent with the mechanism of translational control of GCN4, suggesting that O2 could be a homologue of GCN4 in

maize. The translation of GCN4 is inefficient under normal condition (Hinnebusch, 2005), so the induced GCN4 translation during amino acid starvation causes a drastic change in both GCN4 protein accumulation and GCN4 target gene expression. However, the translation of O2 is not completely inhibited like GCN4 under normal condition, possibly due to the different uORF arrangement in the *O2* transcript. Kozak showed that the efficiency of the uORF inhibitory effect also depends on the transcript sequence upstream of the start codon, notably 3 nucleotides upstream from the AUG (Kozak, 1984a, b). Adenosine was shown to be the most effective nucleotide at the -3 position to favor an AUG codon to serve as an initiation codon, hence the most effective for a uORF to function as an inhibitory barrier (Kozak, 1984a, b). In the *GCN4* transcript, the -3 position of uORF 4 (the uORF that inhibits GCN4 translation) is an adenosine, whereas in *O2* transcript, neither of the uORF 1 or uORF 2 (both uORFs have inhibitory effects of O2 translation) -3 positions is an adenosine (Hinnebusch, 2005; Lohmer et al., 1993). Therefore, the constitutive reinitiation of the *O2* mORF happens regardless of the incubation condition (Figure 3.6B). The less drastic increase of O2 accumulation (~1.4 fold) during amino acid starvation could be explained by the pre-existing baseline expression of O2 and the presence of p-eIF2α under the culture conditions in wild-type endosperm.

In the maize endosperm, the GAAC system is likely to function through ZmGCN2 regulation of O2. Although it is not practical to study transcript expression changes of every possible O2 target, several well-known target genes that were tested exhibited predicted trends. O2 regulates many genes involved in different biological processes, of which ribosome-inactivating protein (RIP) b-32 was a well-known target to be significantly down-regulated when O2 was mutated (Hey et al., 1995). Lysine ketoglutarate

reductase/saccharopine dehydrogenase1 (LKR-SDH1) was also found to be a target of O2 regulation (Jia et al., 2013). Therefore, the changes in gene expression levels of these O2 targets could indicate the changes in the activity of O2 as a transcription activator. qRT-PCR analysis of the amino acid stressed endosperms indicated that the gene expression of *RIP* and *LKR-SDH1* were higher in the stressed endosperms, but not significantly, due to the great variation detected among biological replicates (not shown). To further investigate the transcript expression changes of O2 target genes, it may be necessary to perform a time course study of endosperm tissues incubated on cob culture media to identify an optimal time point for transcript expression analysis.

There might be other targets of GCN2 to accommodate responses to amino acid starvation in other maize tissues, since O2 is endosperm specific and the regulation of GCN2 should not be tissue specific. In other tissues, where a target of ZmGCN2 similar to O2 has not been identified, it is possible to screen for them using bioinformatics approaches. Other possible GCN2 responsive candidates could be indicated using algorithms that scan the entire maize transcriptome for transcripts with highly conserved uORF spacing patterns similar to O2. Sequence analysis has identified 129 transcription associated genes containing uORFs, of which 7 are predicted to function in nucleic acid binding, 15 are involved in responses to biotic and abiotic stresses (Scott and Gibbon, unpublished data). Transcripts of the candidates that are expressed in seedlings will be tested for their responses to GCN2 activated eIF2α phosphorylation by polysome profiling, where the translation states of the candidate mRNAs can be associated with pelleted polysome complexes (Mustroph et al., 2009a; Mustroph et al., 2009b).

One unaddressed question is the involvement of ZmGCN2/O2 in the regulation of amino acid biosynthesis genes. Previous studies have found that the expression levels of several amino acid biosynthesis genes in response to amino acid starvation were not affected by the changes of GCN2 activity (Byrne et al., 2012; Zhang et al., 2008). The exception is *NIA1*, which encodes a nitrate reductase that is responsible for the key step in incorporation of inorganic nitrogen into amino acids. In Arabidopsis, the expression of *NIA1* was significantly reduced in the *gcn2* mutant plants with or without chlorosulfuron treatment (Zhang et al., 2008). Consistently, the expression of *NIA1* was found to be significantly up-regulated in wheat *GCN2* overexpression lines (Byrne et al., 2012), which suggested the role of GCN2 in regulating nitrogen assimilation in plants.

While non-plant eukaryotes use multiple eIF2α kinases to respond to a wide range of biotic and abiotic stresses, plants seem to have a single GCN2 like enzyme as the sole eIF2α kinase. It will be interesting to investigate what other stimuli activate ZmGCN2 and/or phosphorylate ZmeIF2α (such as unfolded protein response, heat, drought, viral infection) and whether maize can use only GCN2 to achieve a similar accommodation to the stressful conditions as the non-plant eukaryotes.

### *Authors' Contributions*

MJ Writing, experimental design and data (All figures and tables included). BCG Experimental design and writing.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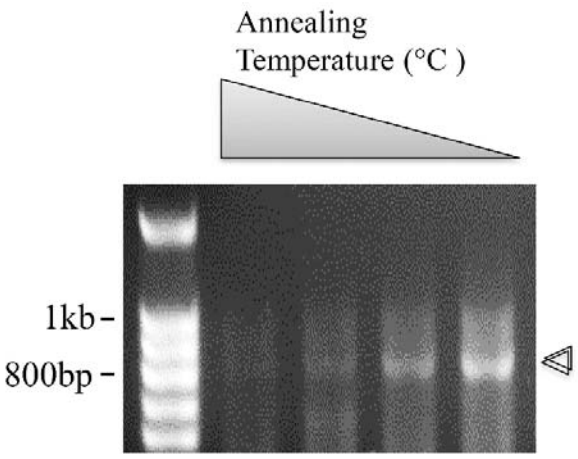
*Supplemental Data*

Supplemental Table S3.1: List of primers used for *Mu* insertion identification and *ZmGCN2* transcript sequencing.

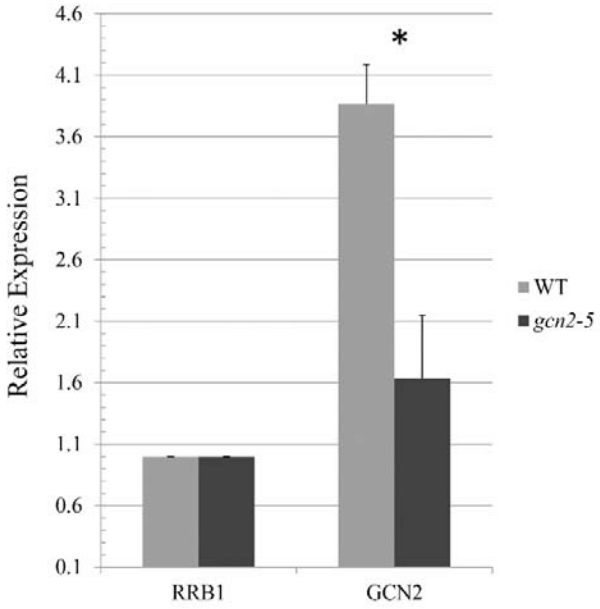
| Primer name (For *ZmGCN2* transcript sequencing) | Forward primer 5' - 3' | Reverse primer 5' - 3' |
|---|---|---|
| ZmGCN2_1 | GCTCTAGAAAGAAAAAGAAGCGCGGC | AAGTTGGAGCTGCCTCCCGT |
| ZmGCN2_2 | TGTTTGAGGCTGCGCAAGAGT | TGCAACACGACCAAAGCCCC |
| ZmGCN2_3 | ACGGGAGGCAGCTCCAACTT | GGTGCCAGGCATGGTCAACATCA |
| ZmGCN2_4 | GGGGGCTTTGGTCGTGTTGCAT | ACCTCGACAGCAGATGGACGGTT |
| ZmGCN2_5 | ACACAGAGACCTGACACCCAGCA | AAAGCGGGTCCCCAGGTCCAAA |
| ZmGCN2_6 | TGCTGTCACCAAGCCCGTCT | TCTCAAACATCTCAGAAGCGCGGA |
| ZmGCN2_7 | TTGGACCTGGGGACCCGCTTTT | TGTGAACACATTGCATCAGACAGGAA |
| ZmGCN2_8 | CGCATGGAACTTGTCGCAGAGC | ACCGCGCACCTTCAGATCCA |
| ZmGCN2_9F | GAACATGTGAAGGCAATCAT | |
| ZmGCN2_10F | GGACCTCTTGATTGTTCTCC | |
| ZmGCN2_5'_1 | AGCTCCAGCTGCTCTAATCG | GATCTTCAAAGCCCATGTCA |
| ZmGCN2_5'_2 | AGATCTCCCTTCCCGTTTGT | TCCTCCCCTAAAATTGAAGC |
| ZmGCN2_5'_3F | CATCCACATCCCAAAGTCC | |
| ZmGCN2_5'_4F | GTCGTGCTTGACTCGAGATG | |

Supplemental Table S3.1, Continued

| Primer name (For *Mu* insertion identification) | Forward primer 5' - 3' | Reverse primer 5' - 3' |
|---|---|---|
| MuTIR6 | AGAGAAGCCAACGCCAWCGCCTCYATTTCGTC | |
| GCN2 screening primer 1 (*gcn2-1, gcn2-2*) | ATCTGACGAGGGCGAAGGTGTC | CAATCAGCAGCGGGAGCACATC |
| GCN2 screening primer 2 (*gcn2-4, gcn2-5*) | AGATCTCCCTTCCCGTTTGT | TCCTCCCCTAAAATTGAAGC |
| GCN2 screening primer 3 (*gcn2-3, gcn2-6*) | TTCTCCTCTCGCCAAGTCAGTTTTAGGC | AGGCAGGATCCGCAACTTTGGACACT |
| Mu TIR 6 | AGAGAAGCCAACGCCAWCGCCTCYATTTCGTC | |
| Primer name (For qRT-PCR) | Forward primer 5' - 3' | Reverse primer 5' - 3' |
| RRB1 | GCTGTTTCTGGTTATGTCTGTCCT | CTTTTGAGTACTTCTGTGCCTGAC |
| GCN2 | GCATGGAACTTGTCGCAGAG | TGAACACATTGCATCAGACAGG |
| O2 | AATCAGCCAGACGCTCGAGATA | ATGAGCTTGCAGTCGCAAGAAA |

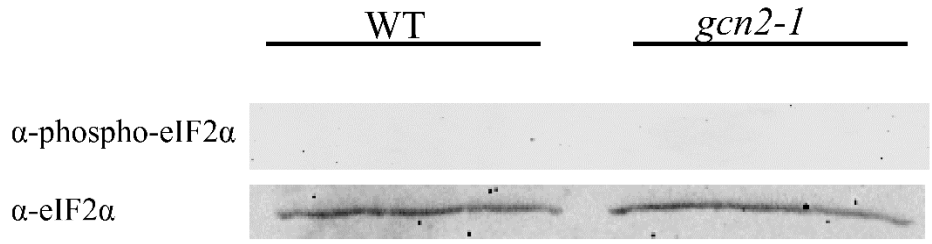



Supplemental Figure S3.1: 3' RACE of ZmGCN2 transcript. The DNA ladder is shown on the left. The positions of the products (854bp and 870bp) are indicated by the arrowheads. The triangle on the top indicates the gradual decrease of the annealing temperature for the 3' RACE (59.7 °C, 58.4 °C, 56.8 °C and 55.6 °C, respectively)

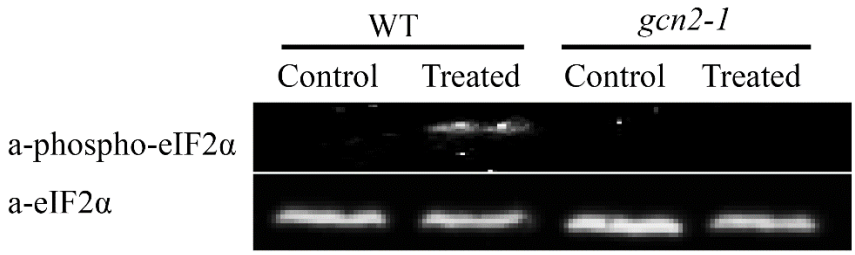



Supplemental Figure S3.2: *ZmGCN2* gene expression levels in 18 DAP endosperms from wild type and *gcn2-5* mutant plants. *ZmGCN2* gene expression decreased significantly in *gcn2-5* mutant 18 DAP endosperm compared to the wild type. The asterisk indicates $p < 0.001$ (n=3).

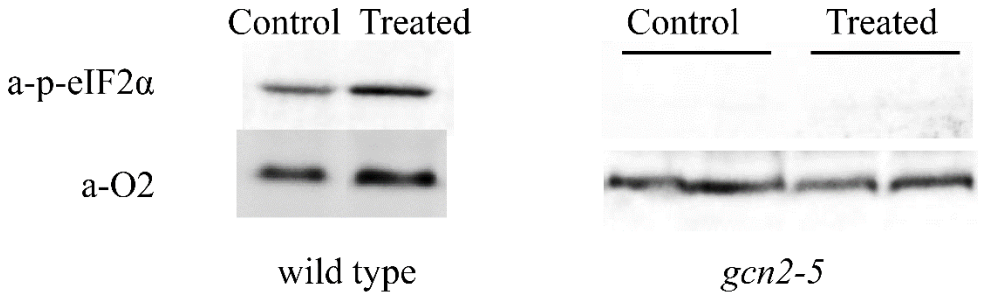

Supplemental Figure S3.3: Multiple alignment of eIF2α serine phosphorylation site by GCN2 (partial). The eIF2α serine phosphorylation site by GCN2 is well conserved among eukaryotes. Identical amino acids at the same position are highlighted in black. The asterisk indicates the phosphorylation site serine 52 by ZmGCN2 kinase domain (Ser 56 in Arabidopsis).




Supplemental Figure S3.4: Western blot analysis of eIF2α from freshly harvested maize endosperms. Both anti-eIF2α and anti-phospho-eIF2α antibodies were used to analyze total soluble proteins extracted from 18 DAP wild type and *gcn2-1* mutant maize endosperms that were frozen upon harvest from the field. There was no phospho-eIF2α detected in either of the genotypes, but similar accumulation levels of eIF2α were detected in both genotypes, suggesting that in normal growth condition, GCN2 induced phosphorylation of eIF2α is minimal.




Supplemental Figure S3.5: Western blot analysis of herbicide treated maize seedlings. After 6 hr of treatment with 20 ppb chlorosulfuron, eIF2α was phosphorylated in wild type seedlings but not *gcn2-1* mutant. This indicates that ZmGCN2 kinase activity is abolished in *gcn2-1* mutant seedlings as well.




Supplemental Figure S3.6: Western blot analysis cob culture treated *gcn2-3* and *gcn2-5* endosperms. No phospho-eIF2α was detected in *gcn2-5* mutant endosperm after herbicide treatment, and there was no change of O2 accumulation either.

# CHAPTER FOUR

## Dissecting the Molecular Mechanisms of General Control Non-Derepressible-2 Regulating Maize Drought Resistance by RNA-Seq


*Abstract*

GCN2 kinase, first discovered in yeast, phosphorylates eukaryotic translation initiation factor 2α (eIF2α) during amino acid starvation. When eIF2α is phosphorylated, it decreases the global translation initiation rate by inhibiting the recycling of eIF2 between successive rounds of protein synthesis, which in turn induces the translation of GCN4 by overcoming the repressing effects of its upstream open reading frames (uORFs). In mammalian cells, there are four eIF2α kinases that respond to different stresses. In plants, GCN2 is the only eIF2α kinase identified and is activated by certain abiotic stresses in Arabidopsis. Previously, maize GCN2 was shown to phosphorylate eIF2α during amino acid starvation and induce the translation of the endosperm-specific transcription factor Opaque2. In this study, we found that gcn2 mutant seedlings were more tolerant to drought stress compared to wild type and the steady state ABA abundance was 2-fold higher in gcn2 mutant leaves. RNA-Seq analysis revealed that ABA biosynthesis genes, such as beta-carotene hydroxylase and zeaxanthin epoxidase, were significantly down-regulated in gcn2 control leaves. There were also fewer genes altered in gcn2 by drought treatment compared to wild type, consistent with the ABA-mediated stress insensitive/tolerant phenotype of gcn2 mutant. Candidate genes that are ABA-responsive were investigated for the potential roles in the increased tolerance in gcn2. Possible causes of elevated ABA abundance in gcn2 and future directions were discussed.

## *Introduction*

When cells are under stress conditions, rapid and efficient regulation of gene expression is essential to regain cellular homeostasis. In cells, translation consumes a substantial amount of cellular energy. Therefore, protein synthesis is usually suppressed during stress (Holcik and Sonenberg, 2005). Post-transcriptional regulation by altering translation is a mechanism that rapidly responds to stresses. There are several regulatory mechanisms that suppress global translation, while promoting the translation of specific regulatory proteins to enable cells to respond to stresses and survive. This translational regulation induces the translation of the preexisting mRNAs and provides an efficient way to control for a large amount of gene expression, because many proteins that are translationally induced through stresses are usually transcription activators (Munoz and Castellano, 2012).

An important step of the aforementioned translational regulation mechanism is the phosphorylation of eukaryotic translation initiation factor 2 α (eIF2α) subunit at serine 51 (Krishnamoorthy et al., 2001). eIF2α is the key component to control translation initiation rate. When phosphorylated, it inhibits the exchange of GDP-bound eIF2 to GTP-bound eIF2, reducing the formation of the translation initiation ternary complex eIF2.GTP.Met-tRNAiMet and negatively regulating the global translation initiation rate (Kimball, 1999).

In mammalian cells, there are several different eIF2α kinases that respond to specific stress signals by reducing translation initiation rate and protein synthesis. Protein kinase R (PKR) responds to viral infection. PKR is activated by binding to double-stranded RNA and inhibits mRNA translation in order to prevent viral protein synthesis (Chou et al., 1995; Williams, 1999). PKR-like endoplasmic reticulum kinase (PERK) responses to

the misfolded protein accumulation in the endoplasmic reticulum (ER), which causes a stress response called unfolded protein response (UPR). PERK is activated and down-regulates protein folding and degradation to reduce the protein load within the ER (Fels and Koumenis, 2006; Harding et al., 2000b). Hemin-regulated inhibitor (HRI) is predominantly expressed in erythroid cells. It is activated in heme deficiency and regulates the synthesis of α- and β-globins in heme-deficient red blood cell precursors (Chen and London, 1995; Han et al., 2001). General control non-derepressible 2 (GCN2) is activated by amino acid starvation (Dever et al., 1993). Although four types of eIF2α kinases have been identified in mammals, GCN2 is the only eIF2α kinase identified in Saccharomyces cerevisiae.

GCN2 is an important regulatory kinase that regulates response to amino acid starvation in eukaryotes. It is activated through interaction with uncharged tRNA, which accumulates during amino acid starvation, and subsequently phosphorylates eIF2α. In yeast, the decrease of translation initiation rate induces the translation of the b-zip transcription factor GCN4 by overcoming the inhibitory effects of the upstream open reading frames (uORF) present in the 5' untranslated region of GCN4 transcript. Therefore, although there is a decrease in general translation initiation, the production of GCN4 protein is increased. GCN4 activates over 30 amino acid biosynthesis genes as well as genes involved in other cellular pathways to help regain homeostasis (Dever et al., 1993; Hinnebusch, 2005).

In plants, GCN2 is considered to be the only eIF2α kinase because no homologues of PKR, PERK or HRI have been identified (Munoz and Castellano, 2012). Studies have shown that phosphorylation of eIF2α could be induced by amino acid deprivation,

wounding, UV radiation, cold shock and hormones involved in defense against insect herbivores (Lageix et al., 2008; Zhang et al., 2008). Although the phosphorylation of eIF2α in response to the stresses appears to be GCN2 dependent, it is not clear whether plants sense and transduce different stress signals through selective translation of specific mRNAs. One of the difficulties to fully characterize the GCN2 kinase pathway in plants is that there are no homologues of GCN4 identified in plants (Halford, 2006).

In our previous study, maize GCN2 was characterized and maize b-zip transcription factor Opaque 2 (O2) was identified as a target of GCN2 regulation during amino acid starvation. Because O2 is expressed specifically in the maize endosperm and phosphorylation of eIF2α was also observed in other tissues, it is speculated that there are other possible targets of GCN2 regulation. eIF2α phosphorylation was induced by GCN2 during amino acid starvation, demonstrating that the pathway was at least partially conserved in maize. In this study, different abiotic stresses other than amino acid starvation were applied to both wild type and *gcn2* mutant seedlings to test whether mutant seedlings were more sensitive to the stressful conditions without GCN2 as a key regulator. Surprisingly, *gcn2* mutant seedlings appeared to be more tolerant to drought treatment compared to the wild type seedlings. There was also a higher steady-state ABA abundance in *gcn2-1* leaves. To better understand the increase of resistance to drought in *gcn2* mutant and to identify candidate genes that are involved in ABA regulation, we performed an RNA-Seq analysis using maize seedling leaves treated with polyethylene glycol (PEG) induced drought stress.

## *Materials and Methods*

### *Plant Materials and Growth Condition*

Maize seeds (*Zea mays L.*) of B73 + and B73 *gcn2-1* were germinated on moist filter paper in the dark at 25°C for 3 days. Germinated seedlings were transferred to pots containing peat moss and topsoil (2:1 by volume). Seedlings were grown at 200 μmol.m−2.s−1 photosynthetically active radiation, a 14/10 h day/ night cycle, a temperature of 25 ºC in a growth room and watered daily.

Drought stress was induced by polyethylene glycol (PEG) using 14-day old seedlings. Uniform seedlings from both genotypes were divided into 2 groups, one group watered with 20% PEG twice, with a 15 min gap, to assure the concentration of PEG in the soil, and the other group supplied with water in the same pattern. Both control and PEG stressed seedlings were harvested at 8 hours after treatment, the third leaves were sectioned, immediately frozen in liquid nitrogen and stored at -70 °C. The second leaves were saved to measure the ABA accumulation levels.

For long-term effects, the seedlings were watered normally until 14-day old and then drought stress was applied by either withholding water for 7 days or watering with 20% PEG for 4 days.

### *Quantitative Real-Time PCR Analysis (qRT-PCR)*

qRT-PCR was performed as described previously (Jia et al., 2013). RNA was isolated from maize endosperm at 18 days after pollination (DAP). cDNA was synthesized with qScript™ cDNA SuperMix (Quanta Biosciences, Gaithersburg, MD) and qRT-PCR was performed using PerfeCTa SYBR Green FastMix (Quanta Biosciences, Gaithersburg,

MD) on Corbett Rotor-Gene™ 3000 (Qiagen, Velancia, CA). Primers used in qRT-PCR were as follows: *CIPK17* forward: 5'- AGGTTACGAGTCACCTGATGAGC; *CIPK17* reverse: 5'- ATCGTAAGGAGACAACGATCCAA.

*Measurement of ABA Abundance*

Maize leaf tissues collected from the drought treatment were lyophilized and ground to fine powders. Five mg of leaf powders (dry weight) were accurately measured and subject to ABA purification. ABA abundance was quantified using isotope dilution selected ion monitoring gas chromatography-mass spectrometry [GC-MS; Reddy, et al, 2013]. Briefly, 1 ng stable isotope-labeled [2H6] ABA was added to each sample replicate and samples were extracted with 500 µL 55°C methanol twice and 500 µL 55°C 80% ethanol once, with the cleared supernatants collected after each extraction and centrifugation. The extracts were dried and resuspended with 800 µL chloroform, followed by mixing with 1mL H2O adjusted to pH 9.0 with NH4OH. The aqueous fraction was collected and mixed with 1mL ethyl acetate adjusted to pH 5.0 with acetic acid. The organic fraction was dried and methylated with ethereal diazomethane. Samples were analyzed on an Agilent 7890A/5975C XL GC-MS with a 0.25 mm x 30 m DB-5MS column using pulsed splitless injection (7693A). The flow rate of the helium carrier gas was 0.75 mL/min. The inlet temperature was 250 °C. The oven temperature ramped from 45°C to 250°C at 40°C/min, held at 250°C for 3 min, and then again ramped to 290°C at 40°C/min. The total run time was 9.125 min. The electron impact source and quadrupole mass analyzer were maintained at 230 °C and 150 °C, respectively. Data was obtained in both scan and selective ion monitoring. One quantitation ion and one qualitative ion were monitored for each hormone (i.e. ABA, 190 and 194 mass-to-charge ratio).

*RNA Isolation, cDNA Library Construction and RNA-Sequencing*

Total RNA was isolated from pooled leaves and roots of 5 individuals for each genotype and each treatment using the Trizol reagent (Invitrogen, Grand Island, NY) and then cleaned up with the RNeasy Plant Mini Kit (Qiagen, Valencia, CA) with an on column DNase treatment (Qiagen, Valencia, CA) according to the manufacturer's instructions. Two biological replicates were used for each treatment. The quality of total RNA was determined by a 2100 Bioanalyzer (Agilent Technologies, Inc, Santa Clara, CA) and the libraries were prepared by the Illumina TruSeq Stranded mRNA sample prep kit (Illumina, San Diego, CA). Adapter ligated libraries were quantified by quantitative PCR using the Kapa Library Quant Kit (Kapa Biosystems, Woburn, MA) and the concentration was further adjusted to prepare the libraries for Illumina sequence analysis. RNA-Sequencing was performed on Illumina HiSeq 2000 with 50 cycle single end read sequencing by the Microarray and Genomic Analysis Core Facility at the University of Utah. Approximately 20 million reads were generated for each sample.

*RNA-Sequencing Data Processing*

The RNA-Seq reads were processed to remove the adapter sequences and the quality of the trimmed reads were evaluated with FastQC (version 0.10.1) (www.bioinformatics.babraham.ac.uk/projects/fastqc/). The dataset was determined to be of high quality (quality scores were over 30 for all samples). It was then processed through the Tuxedo2 RNA-Seq analysis package provided by iPlant Discovery Environment (Oliver et al., 2013). Sequences were mapped to the maize B73 reference genome (Assembly version: AGPv3) using TopHat2 (Trapnell et al., 2009) with default parameters, except that the strand information was chosen to be first strand. Over 90% of the reads

were mapped to the reference genome in all the samples. The TopHat2 generated alignments were then assembled using Cufflinks2 (Trapnell et al., 2010). The gene abundance was determined using Cufflinks by calculating the fragments per kilobase of exon per million fragments mapped (FPKM) value. In order to calculate fold changes with log adjustment, genes with 0 FPKM are assigned a value of 0.002 (Rowley et al., 2011). Cuffmerge2 (Trapnell et al., 2010) was then used to merge the assemblies with the maize B73 reference genome annotation into a single GTF file that was used in Cuffdiff2 (Trapnell et al., 2010) to identify differentially expressed genes. Since there were two biological replicates for each genotype/treatment, eight assemblies were generated for each tissue. Only four assemblies representing two treatments or genotypes were merged and processed through Cuffdiff2 to avoid increasing the degree of freedom and subsequently causing increased false positive results (i.e. wild type leaf control compared to wild type leaf treated, *gcn2-1* root control vs *gcn2-1* root treated, etc). Differentially expressed genes were determined if 1) FPKM for a gene was greater than 1 in at least one of the two genotype/treatments being compared 2) it was part of the maize Filtered Gene Set (FGS) 3) the false discovery rate (q value) was less than 0.05 and 4) the fold change is greater than 2 (Bi, et al, 2014). The results were visualized using the cummeRbund package (Trapnell et al., 2012).

*Gene Ontology (GO) Enrichment*

GO functional annotations were determined by AgriGO (Du et al., 2010) and used to assess the biological functionality of candidate genes. Singular enrichment analysis was used to test the significance against the suggested background maize genome *zea mays* ssp V5a. The minimum mapped entries were set at five, thus only five or more GO annotations

that appeared for one analysis would be shown. Fisher distributions were applied to test the significance against background under the maize genome. The p-values were adjusted for multiple testing by controlling false discovery rate with the Hochberg method.

## *Results*

### *gcn2 Mutant Seedlings are More Tolerant to Drought Stress*

In standard conditions, B73 *gcn2-1* seedlings grew normally compared to the wild type. When treated with herbicides that inhibit amino acid biosynthesis, B73 *gcn2-1* seedlings were more sensitive to amino acid starvation compared to wild type seedlings (not shown), consistent with the reports in other organisms (Zhang et al., 2002; Zhang et al., 2008). In 2011, the state of Texas encountered extreme high temperatures and severe drought in the summer (Achberger et al., 2012). Surprisingly, B73 *gcn2* mutants appeared to be less affected by the harsh conditions and grew healthy, whereas B73+ plants displayed slowed growth and wilting and were substantially shorter in stature at flowering (Figure 4.1A, *gcn2-1* mutants had better field performance than B73+ in the summer of 2013). *gcn2-1* mutant kernels were slower to germinate compared to the wild type by 1 or 2 days (Figure 4.1B).

The molecular basis for the differences in stress tolerance was further investigated by analyzing expression of genes shown to convey stress tolerance in other systems. Interestingly, the transcript level of at least one calcineurin B-like protein (CBL)-interacting protein kinase family (CIPK), *CIPK17*, was constitutively increased in the mutant endosperms (Figure 4.2, $p < 0.001$, n=3). CBLs are a multigene family of calcium sensors and several CBLs were found to play roles in response to abscisic acid (ABA)

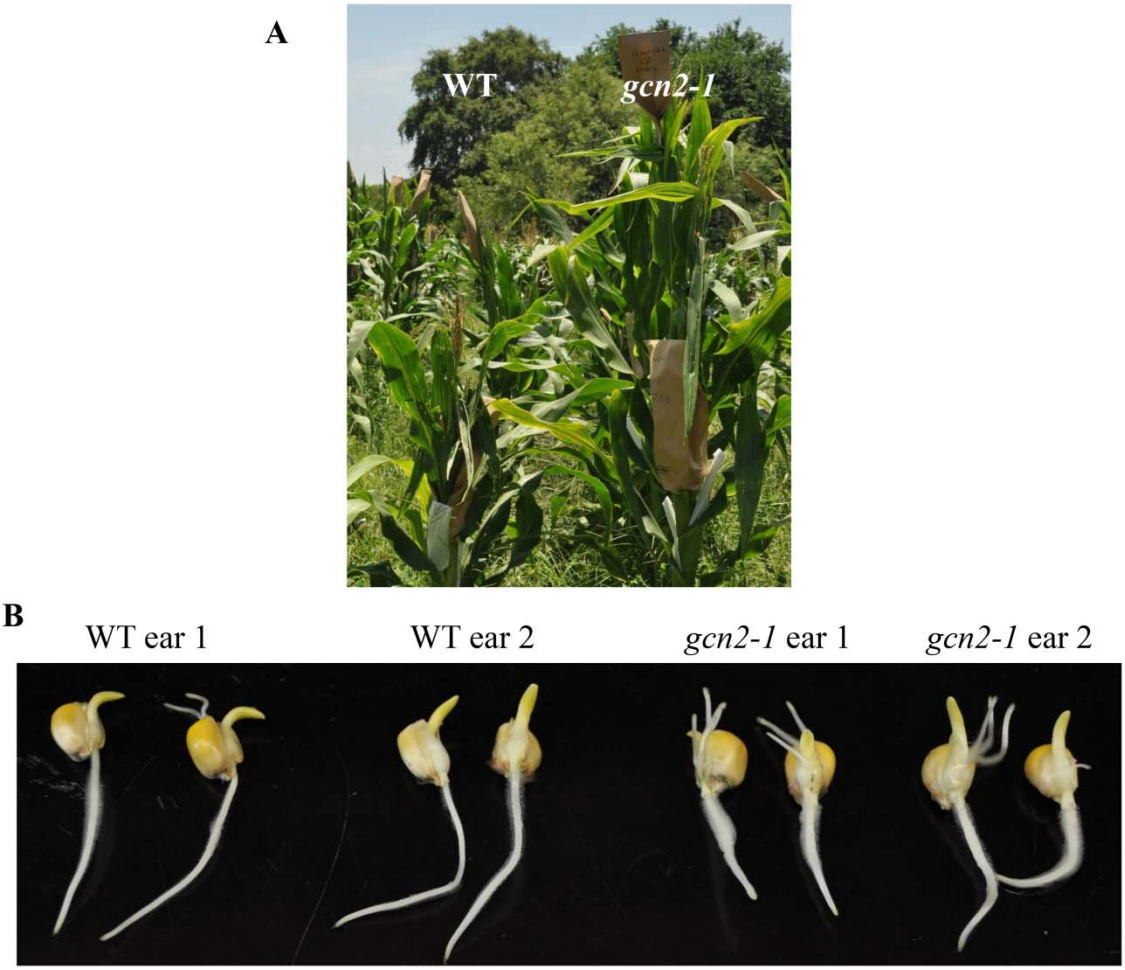



Figure 4.1: Maize B73 *gcn2-1* mutants exhibit phenotypes indicating altered ABA accumulation. A) *gcn2-1* mutant plants had better performance in the field during heat and drought, compared to wild type. B) *gcn2-1* mutant kernels are slower to germinate compared to wild type kernels.

mediated stresses and interacted with a group of serine/threonine protein CIPKs to activate downstream cascades that leads to altered expression of stress-induced genes (Chen et al., 2011; Cheong et al., 2003; Luan, 2009; Luan et al., 2002; Xiang et al., 2007; Zhao et al., 2009). It is reported that there are at least 43 putative CIPK genes in the maize genome;

they function in both independent and redundant ways in response to various abiotic stresses (Chen et al., 2011). However, other CIPK or CBL genes tested did not indicate significant alterations in *gcn2-1* mutants (data not shown).

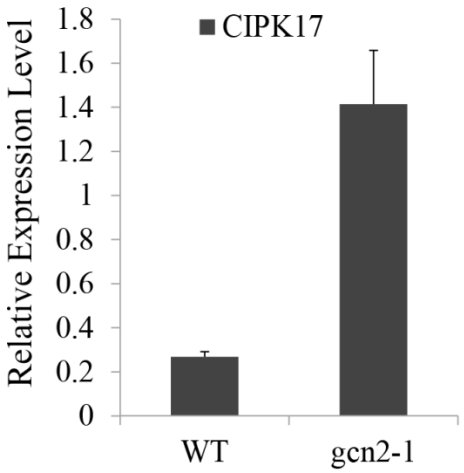



Figure 4.2: qRT-PCR analysis of the transcript level of *CIPK17* in maize 18 DAP endosperms. *CIPK17* is significantly up regulated in *gcn2-1*. $p < 0.001$, n=3.

The improved field performance, delayed germination and increased *CIPK17* expression level in *gcn2-1* mutants indicated that ABA responses are altered in *gcn2-1* mutants. These observations support a model that GCN2 may play a role in ABA accumulation in plants and may regulate ABA-mediated abiotic stress responses, such as drought.

To investigate the role of GCN2 in maize response to drought, maize B73 wild type and *gcn2-1* mutant seedlings were grown in soil for 14-days and treated with 20% polyethylene glycol (PEG) for 8 hrs. Second leaves were harvested and analyzed for ABA abundance by GC-MS. Third leaves and roots were collected and used for RNA-Seq analysis. Figure 4.3 shows that ABA level increased by 4.70-fold in wild type leaves after

PEG treatment ($p < 0.001$, n=7), whereas in *gcn2-1* mutant leaves, the ABA level only increased by 1.70-fold after PEG treatment ($p< 0.05$, n=7). Pairwise comparison revealed that the endogenous abundance of ABA was significantly higher in *gcn2-1* leaves and almost doubled the abundance in the wild type leaves when growing in normal conditions ($p< 0.001$, n=7). However, the abundance of ABA appeared to have no significant difference after PEG treatment between wild type and *gcn2-1* mutant leaves ($p=0.14$, n=7). Although the variances among individual samples could potentially mask the differences between the two genotypes after PEG treatment, it was clear that the steady-state endogenous ABA level is significantly higher in *gcn2-1* mutants. However, the *gcn2-1* mutant appeared to be less responsive to drought stress, as indicated by the lower fold-change increase of ABA after treatment.

Long term drought treatment was applied to 14-day-old seedlings by water withholding for 7 days or 20% PEG treatment for 4 days. Consistent with the observations from the field, wild type seedlings appeared to be under stress, as indicated by slower growth, curled leaves and brown leaf tips. In contrast, *gcn2-1* mutant plants were less affected compared to the control plants (Figure 4.4A). *gcn2-5* mutants were used as a second allele to confirm the findings in gcn2-1 mutants and it is nearly isogenic to maize inbred line W22. *gcn2-5* is also a GCN2 knockout allele but has a different Mutator insertion site than *gcn2-1*. After PEG treatment, it also showed less leaf curling and growth inhibition than wild type (Figure 4.4B). The seedling size differences between WT and *gcn2-5* could be explained by the higher kernel mass of W22 wild type compared to *gcn2-5*.

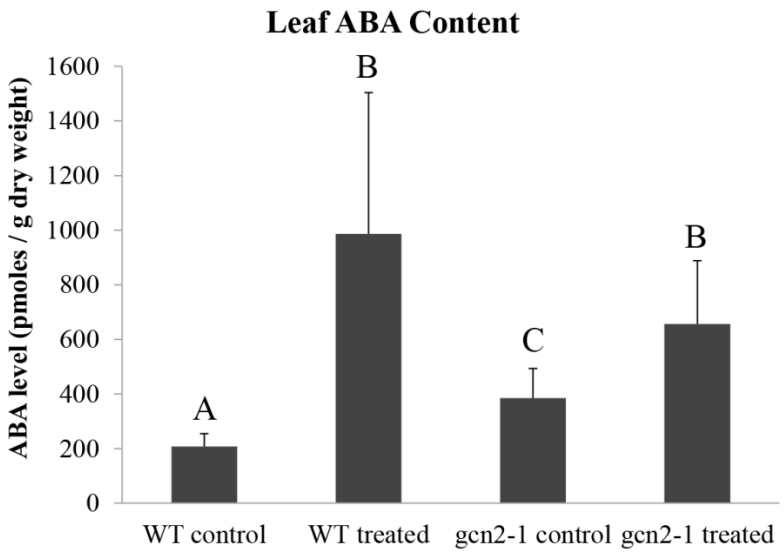



Figure 4.3: Measurement of ABA level in maize leaves. A, B and C indicate significant differences $p<0.05$ (n=7).

*RNA-Seq Analysis of Maize Leaf Transcriptome in Drought Stress*

To better understand the role of GCN2 in maize drought responses and to identify candidate genes that are regulated by GCN2 and involved in ABA-related physiological pathways, an RNA-Seq analysis was performed. The third leaves were collected from uniform seedlings and were subjected to RNA-Seq. There were 8 samples in total and each condition had two biological replicates that were pooled from five uniform seedlings to control for individual variances. There were approximately 160 million reads of 50 nucleotides in length generated, with an average of 20 million reads from each sample. Figure 4.5 diagrams the workflow of the RNA-Seq analysis. Reads were mapped to the maize reference genome with TopHat2, and then a transcriptome assembly for each condition was generated by Cufflinks2. These assemblies were merged together by Cuffmerge2 to form a uniform basis for calculating gene and transcript expression levels in each condition. Cuffdiff2 was used to calculate expression levels and test the statistical

differences of changes in expressions. Finally, CummeRbund was used to create expression plots and figures (Trapnell et al., 2012). The reads generated in this study will be uploaded to the NCBI sequence read archive (SRA).

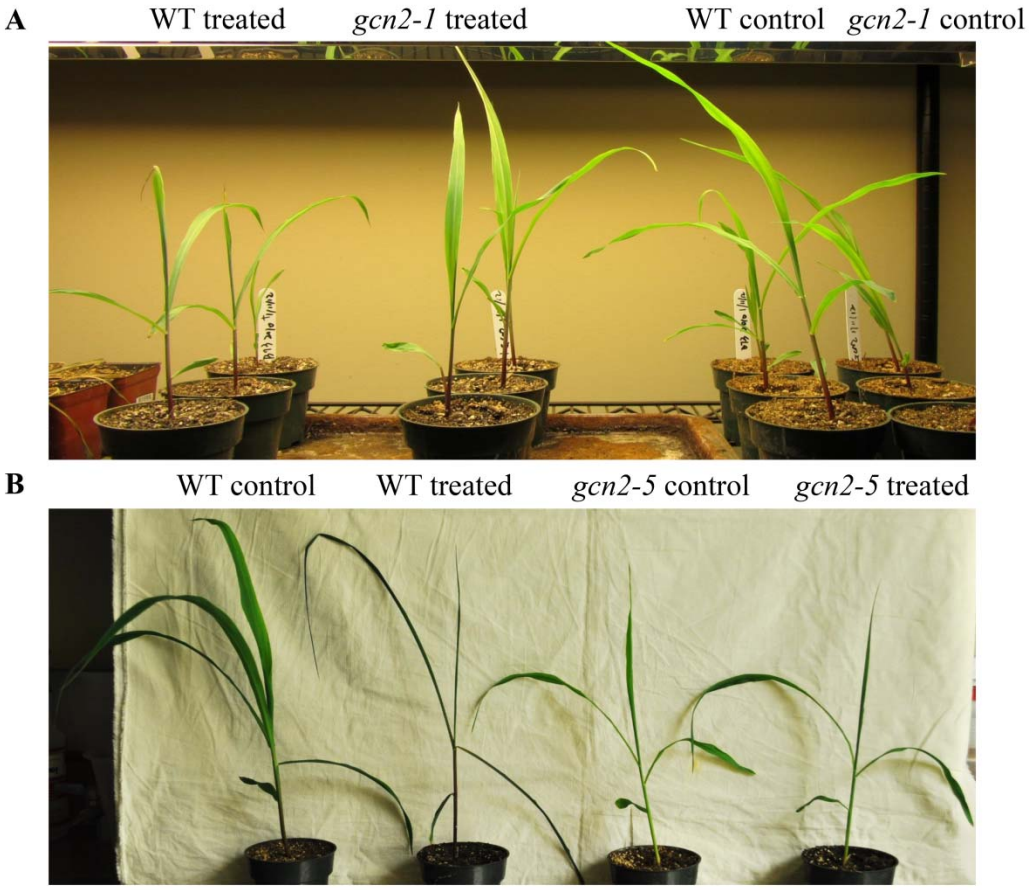



Figure 4.4: Drought treatment of wild type and *gcn2* seedlings. A) *gcn2-1* seedlings appeared to be less sensitive to drought treatment. B) *gcn2-5* seedlings were less affected by drought compared to wild type.

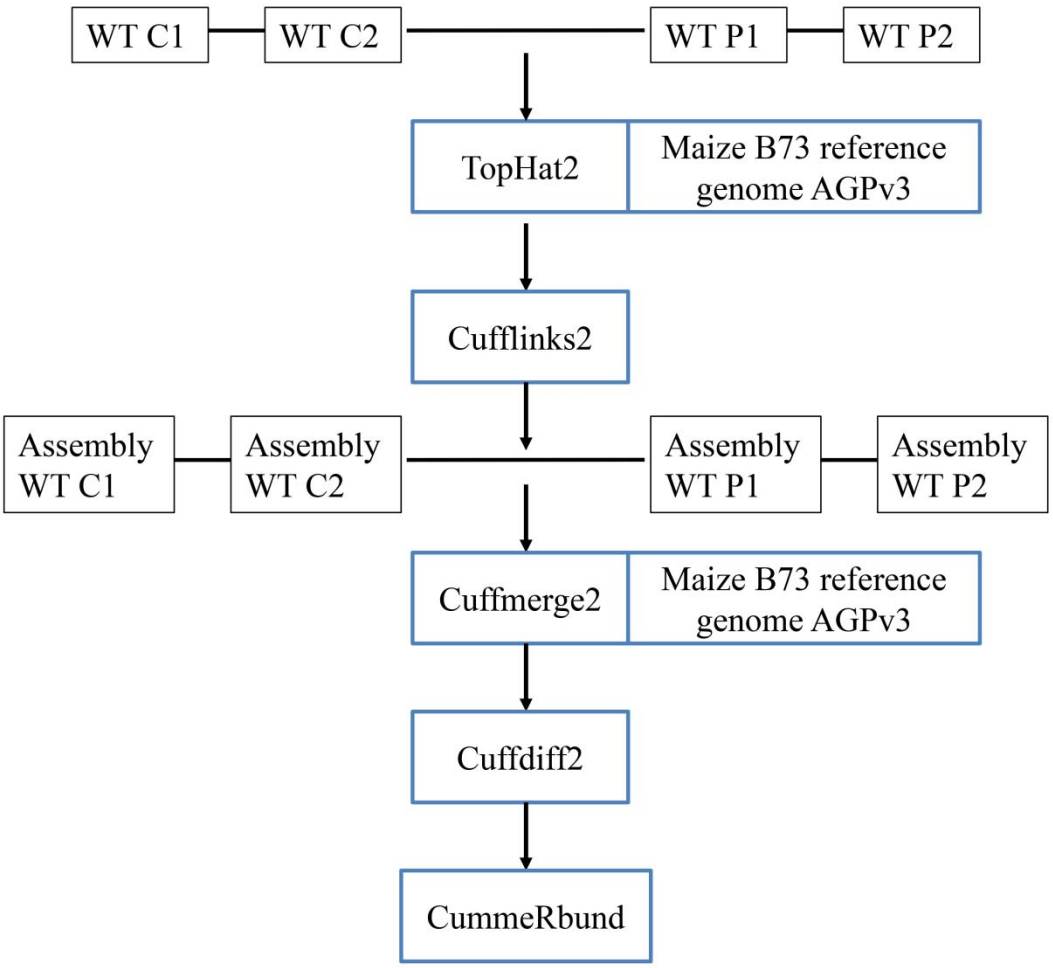



Figure 4.5: The workflow of RNA-Seq in this study. WT C1 represents wild type control replicate 1. WT P1 represents wild type PEG treated replicate 1.

Of the 0.15 billion reads generated by RNA-Seq, 94.6% were be mapped to the maize B73 reference genome by TopHat2. The transcript abundance for each gene was estimated by fragments per kilobase of exon per million fragments mapped (FPKM) using Cufflinks2. To identify differentially expressed genes between conditions, a very stringent cutoff was applied to the Cuffdiff2 output as described in Materials and Methods. For comparisons of genes that were altered by the PEG treatment (wild type control vs wild type treated and *gcn2-1* control vs *gcn2-1* treated), 830 genes were found to be differentially expressed in response to the PEG induced drought treatment in wild type

leaves; substantially fewer genes were found to have altered expression levels after treatment in *gcn2-1* mutant tissues with 228. In the 830 differentially expressed genes identified in wild type tissues, 703 genes were up-regulated in leaves whereas 127 genes were down-regulated. In the 228 differentially expressed genes identified in the *gcn2-1* mutant tissues, 204 genes were up-regulated in leaves whereas 24 genes were down-regulated in leaves. 102 genes were found to be significantly altered in expression levels by PEG treatment only in *gcn2-1* leaves (Supplemental Figure S4.1). For comparisons of genes that were altered by the *gcn2* mutation (wild type control vs *gcn2-1* control and wild type treated vs *gcn2-1* treated), 1536 genes were differentially expressed in *gcn2-1* control leaf compared to wild type leaf tissues; 1366 genes were altered in *gcn2-1* treated leaf compared to wild type leaf tissues.

Density plots of log-transformed FPKM values of the expression level distribution for all genes in compared conditions suggested that the distribution patterns of samples being compared for differential expression are almost identical (Supplemental Figure S4.2). Consistent with density plots, scatter plots also showed general similarities between compared conditions, and the outliers could be identified as well (Supplemental Figure S4.3). Volcano plots highlighted genes that were significantly altered between compared conditions (Figure 4.6).

*GO Enrichment Analysis of Differentially Expressed Genes*

GO enrichment analysis of differentially expressed genes between two conditions was performed by AgriGO (GO analysis toolkit and database for agriculture community; http://bioinfo.cau.edu.cn/agriGO/index.php). Singular enrichment analysis was used with maize genome build V5a . A list of candidate gene IDs was provided to AgriGO to be

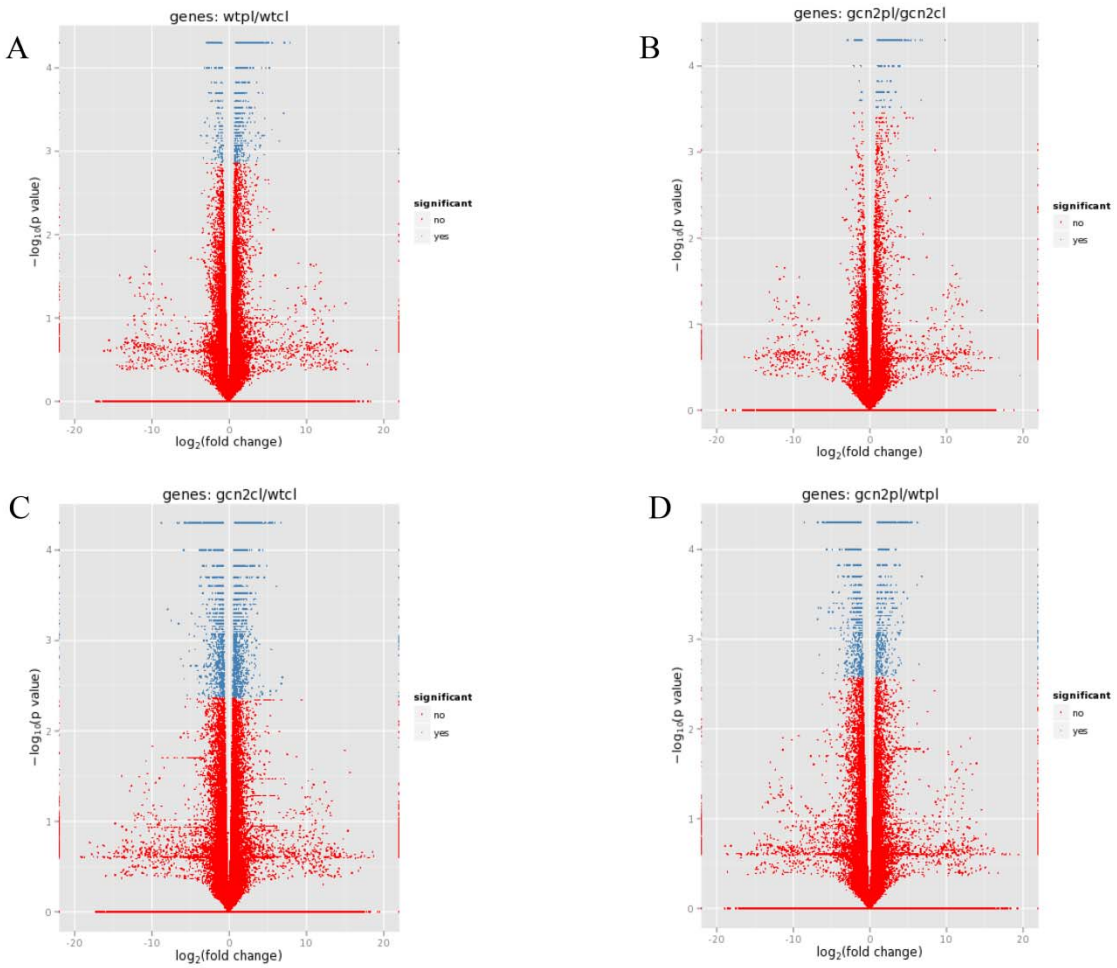



Figure 4.6: Volcano plots of different comparisons between conditions. A) wild type control and treated. B) gcn2-1 control and treated. C) wild type control and gcn2-1 control. D) wild type treated and gcn2-1 treated. Differentially expressed genes between compared conditions were highlighted in blue.

assigned for GO IDs, which were then mapped to the plant GO slim. There were 8 significant GO terms showing enrichment between wild type control leaves and treated leaves compared to the B73 reference as the background, including 6 GO terms involved in biological processes (Figure 4.7) and 2 GO terms for molecular function. The enriched terms for biological processes were carbohydrate metabolic process (GO: 0005975), metabolic process (GO: 0009056), cellular amino acid and derivative metabolic process (GO: 0006519), secondary metabolic process (GO: 0019748), and response to stimulus

(GO: 0050896). For molecular functions, catalytic activity (GO: 0003824) and transferase activity (GO: 0016740) were enriched. A detailed comparison of overrepresented GO terms involved in wild type leaf drought response to background is shown in Supplemental Figure S4.4.

For *gcn2-1* leaves, PEG treatment induced enrichment of 6 GO terms, including 5 in biological processes (Figure 4.8) and 1 in molecular function. The enriched GO terms for biological processes were carbohydrate metabolic process, response to stimulus (GO: 0050896), response to stress (GO: 0006950), response to abiotic stimulus (GO: 0009628), and multicellular organismal process (GO: 0032501). Catalytic activity was the only molecular function enriched. Pairwise comparison revealed that while there were many GO terms enriched comparing *gcn2-1* control leaves to wild type (Figure 4.9), only biological processes such as death (GO: 0016265), cell death (GO: 0008219) and metabolic process were enriched when comparing *gcn2-1* treated leaves to wild type (Figure 4.10) . Overrepresented GO terms for the comparisons mentioned above are shown in detail in Supplemental Figures S4.5, S4.6 and S4.7.

Candidate genes associated with the GO terms “response to stimulus”, “response to stress”, and “response to abiotic stimulus” were further investigated.

*Differentially Expressed Genes in Response to Stress*

There were 48 candidate genes involved in biological processes such as response to stress in leaf tissues at least in one genotype after PEG treatment. A heat-map representation shows the fold change values of candidate gene expression levels (Figure 4.11). Many similar genes were up-regulated by PEG treatment in both genotypes, such as ABA responsive protein (GRMZM2G106622), beta-amylase (GRMZM2G450125), beta-

carotene hydroxylase (GRMZM2G382534), dehydrin 1 (DHN1, also known as Rab17, GRMZM2G079440), dehydrin 2 (DHN2, GRMZM2G373522), ethylene-responsive protein (GRMZM2G059799), NaCl stress protein1 (GRMZM2G015605), nonspecific lipid-transfer protein (GRMZM2G387360), and viviparous 14 (VP14) (GRMZM2G014392). Although the fold-changes of gene expression were sometimes different between genotypes, many of these genes are well known to change expressions in response to drought. DHNs belong to the group 2 late embryogenesis abundant (LEA) proteins, and they are induced during the late stage of embryogenesis or by drought, freezing or ABA application (Close, 1996). Both wild type and *gcn2-1* leaves showed substantial increases in *DHN1* and *DHN2* transcript levels after PEG treatment, and the increase of *DHN1* was 4 fold higher in *gcn2-1*. VP14 is a 9-cis-epoxycarotenoid dioxygenase (NCED) that catalyzes the rate-limiting step in ABA biosynthesis, which is the oxidative cleavage of the 11, 12 double bond of a 9-cis-epoxycarotenoid (Tan et al., 1997). It is reported that overexpression of *VP14* increases drought tolerance in *Nicotiana plumbaginifolia* (Qin and Zeevaart, 2002). The abundance level of *VP14* was also increased to 4-fold higher in *gcn2-1*. However, another NCED, *NCED5* (GRMZM2G417954), the homologue of *AtNCED9*, was only up regulated in the wild type. *AtNCED9* has been reported to regulate ABA synthesis in the embryo and endosperm and function in seed dormancy, but it is not induced by drought stress in *Arabidopsis* seedlings (Frey et al., 2012; Iuchi et al., 2001).There were also candidate genes that were down-regulated by PEG in both genotypes, such as an unknown protein

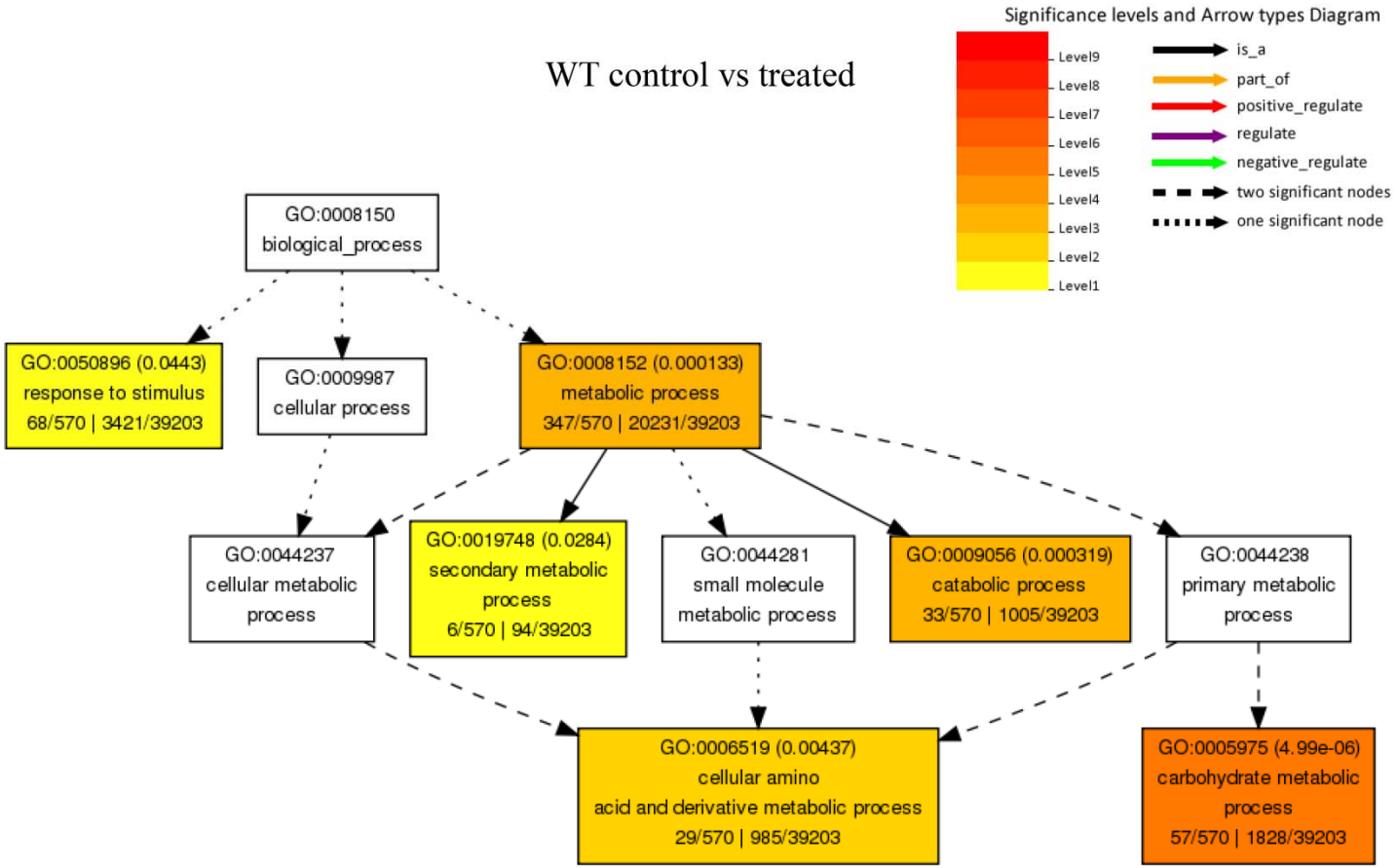



Figure 4.7: GO enrichment analysis of genes altered by PEG treatment in wild type leaves displayed in the hieratical pattern.

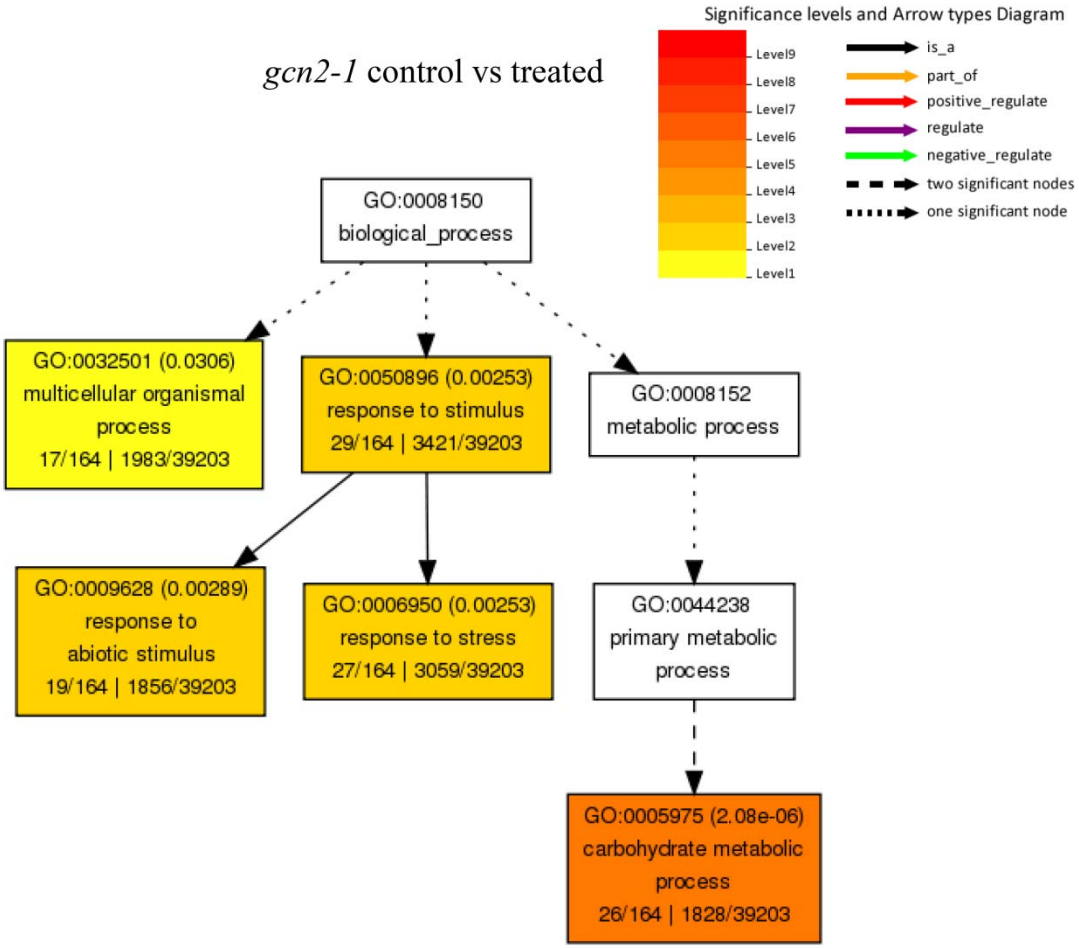



Figure 4.8: GO enrichment analysis of genes altered by PEG treatment in gcn2-1 leaves displayed in the hieratical pattern.

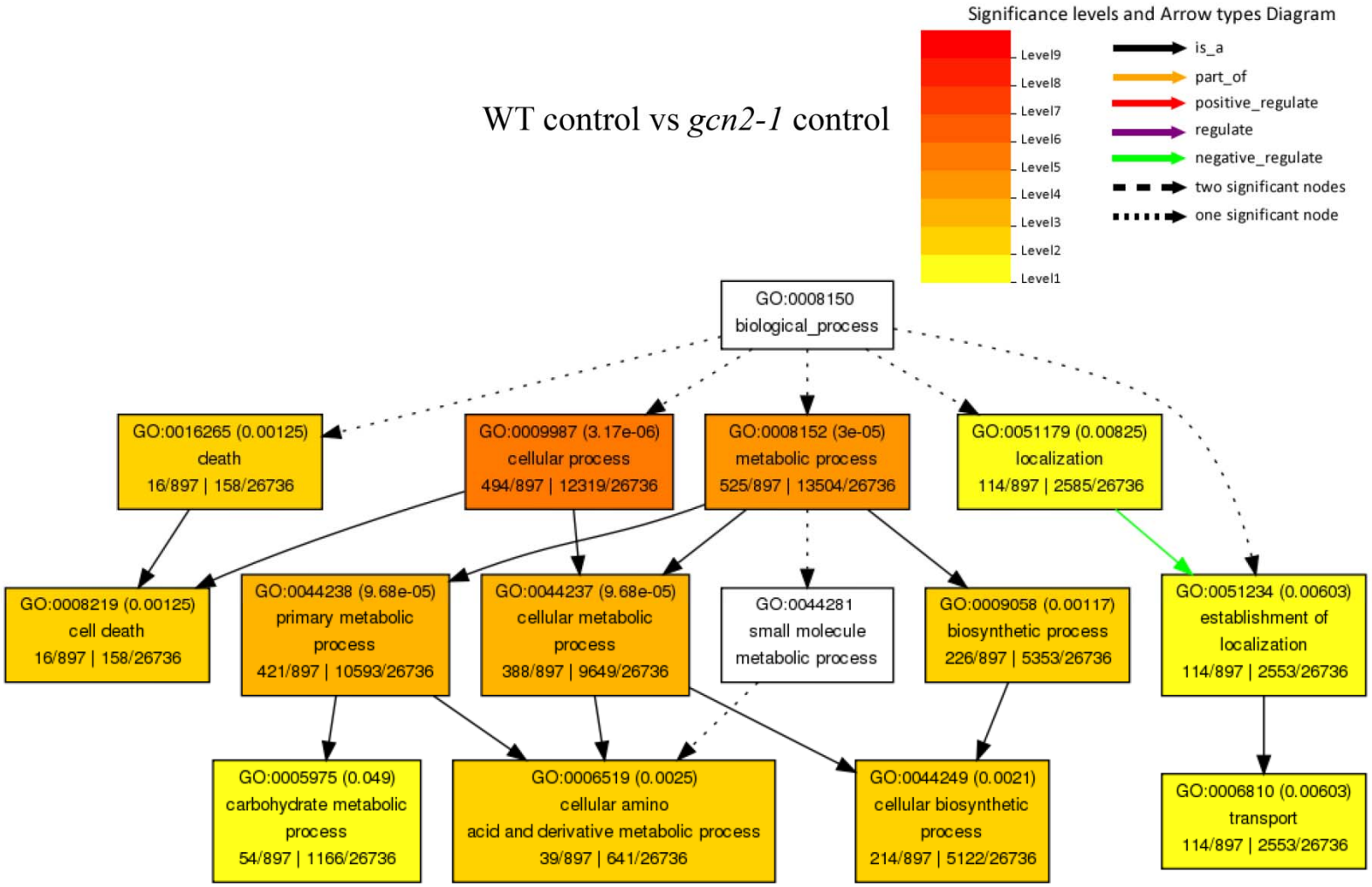



Figure 4.9: GO enrichment analysis of genes that are differentially expressed between wild type and gcn2-1 control leaves displayed in the hieratical pattern.

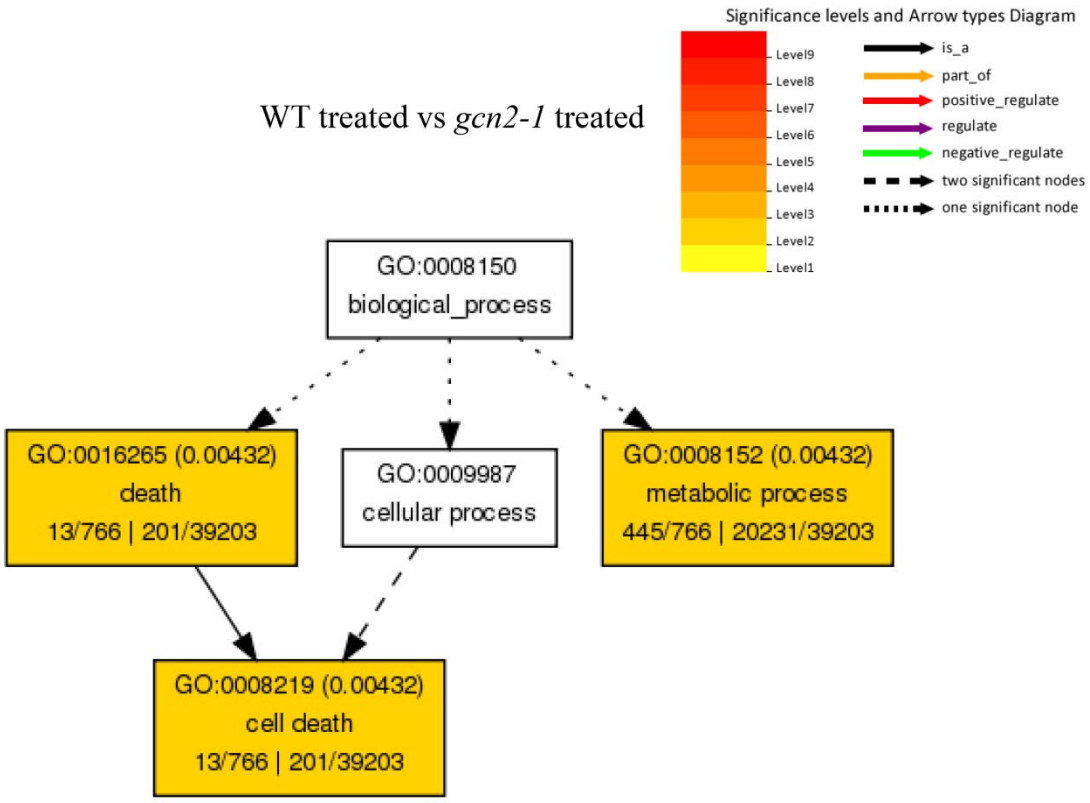



Figure 4.10: GO enrichment analysis of genes that are differentially expressed between wild type and gcn2-1 treated leaves displayed in the hieratical pattern.

that is involved in response to stress (GRMZM2G136158). Genes encoding proteins that are induced by elevated ABA abundance or drought, such as drought-induced protein 1 (GRMZM2G098460), embryonic abundant protein-like protein (GRMZM2G148904), late embryogenesis abundant protein, group 3 (GRMZM2G425629) and wound/stress protein (GRMZM2G087245), showed significant increases in transcript abundance in the drought-stressed wild type leaves, whereas no changes were observed in the drought-stressed *gcn2-1* leaves. The group 3 LEA proteins accumulate in late embryogenesis as well as in dehydrated seedlings, and can be induced by ABA or drought (Curry et al., 1991). They are hydrophilic and function in protecting seedlings from desiccation (Ried and Walker-Simmons, 1993). Interestingly, the transcript abundance of *LEA* only increased in wild type

leaves, indicating that the *LEA* abundance in *gcn2-1* is insensitive to changes in ABA levels. Pairwise comparisons of wild type and *gcn2-1* leaf tissues indicated that the abundance of *LEA* transcript was 2 to 3 fold lower in *gcn2-1* leaf tissues regardless of treatment (described below). On the contrary, drought-responsive element (DRE) binding factor 1 (DBF1, GRMZM2G061487) was up regulated by PEG treatment only in *gcn2-1*. DREs, DRE2 in particular, are *cis*-elements that regulate the promoter of rab17 after water stress or ABA application (Busk et al., 1997). DBF1 was reported to interact with DRE2 *cis*-element of the rab17 promoter in maize and DBF1 can be induced by ABA and dehydration (Kizis and Pages, 2002). CIPK16 (GRMZM2G137569), induced by ABA and dehydration as well (Zhao et al., 2009), was also shown to be up regulated only in *gcn2-1*.

Pairwise comparison between wild type and *gcn2-1* leaf tissues indicated that several ABA inducible genes were significantly decreased in *gcn2-1* control leaf (Figure 4.12). CIPK11 (GRMZM2G177050) is up regulated by ABA, drought and salinity in *Arabidpopsis* (Fuglsang et al., 2007), but a significant increase of *CIPK11* transcript by drought was not detected in maize (Chen et al., 2011). *LEA* was also down regulated in *gcn2-1* control leaf tissues. On the contrary, an AP2/EREBP transcription factor (GRMZM2G104260) was significantly up regulated in *gcn2-1* control leaf compared to the wild type. AP2/EREBP-type proteins are transcription factors that recognize DRE in the promoters (Yamaguchi-Shinozaki and Shinozaki, 1994), and could be induced by abiotic stresses such as drought and salinity (Kizis and Pages, 2002). Sucrose non-fermenting protein (SNF1) related protein kinase (SnRK , GRMZM2G130018) was also

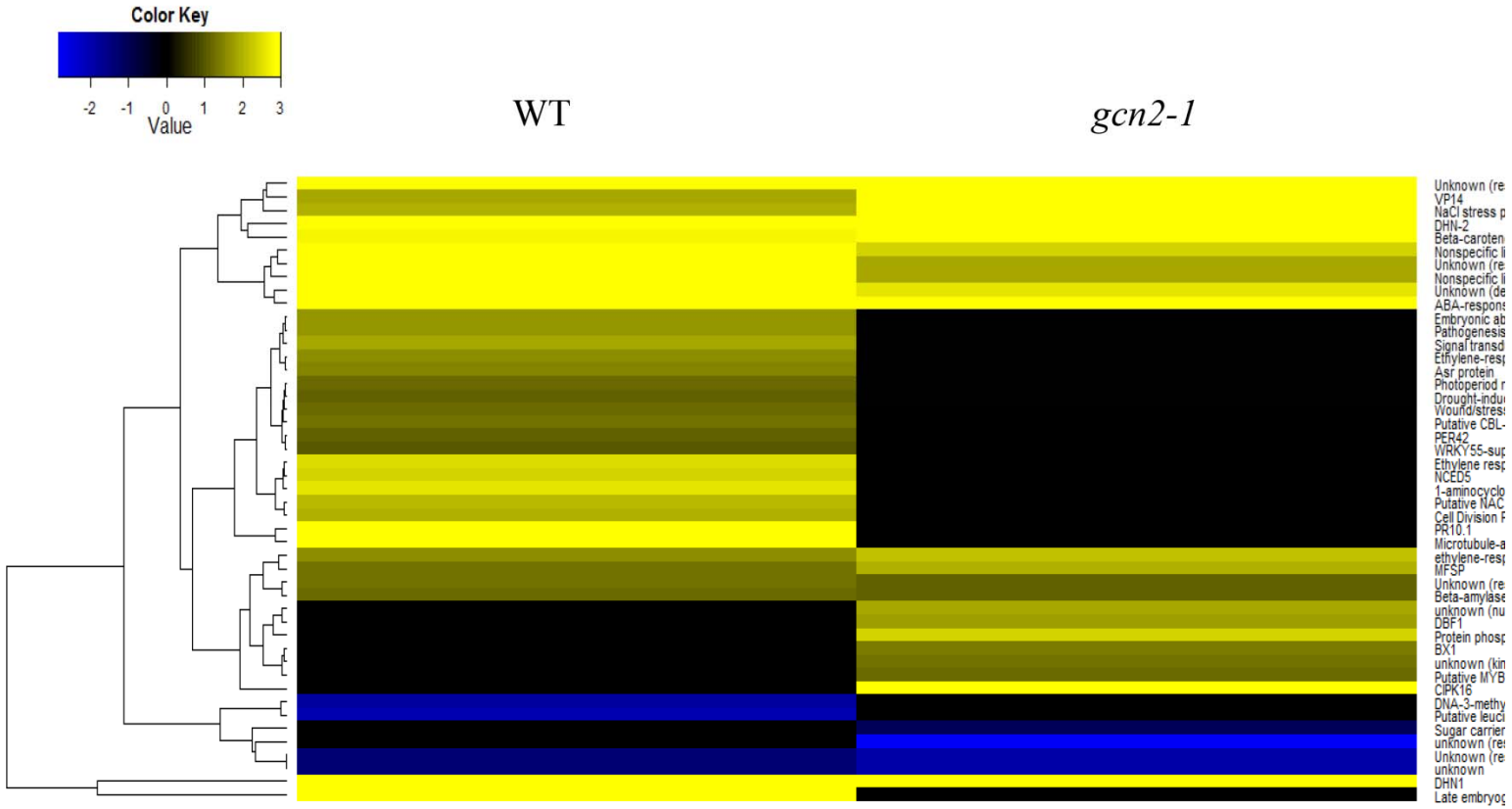



Figure 4.11: A heatmap representation of genes that are significantly altered by PEG treatment in both genotypes.

significantly up regulated in *gcn2-1* control leaves. In *Arabidopsis*, SnRK1 has a central role in ABA signaling, and overexpression of SnRK1 is hypersensitive to ABA (Jossier et al., 2009). There were several genes that were up regulated to 3-4 fold higher after PEG treatment in *gcn2-1* leaves compared to the wild type, such as CIPK16, VP14 and DHN1.

*Discussion*

The surprising observation of the higher drought tolerance of *gcn2-1* mutant seedlings led to further investigation of the molecular mechanisms underlying the response. Because *gcn2-1* mutant kernels showed delayed germination and had significantly higher transcript levels of at least one of the CIPK gene family, which

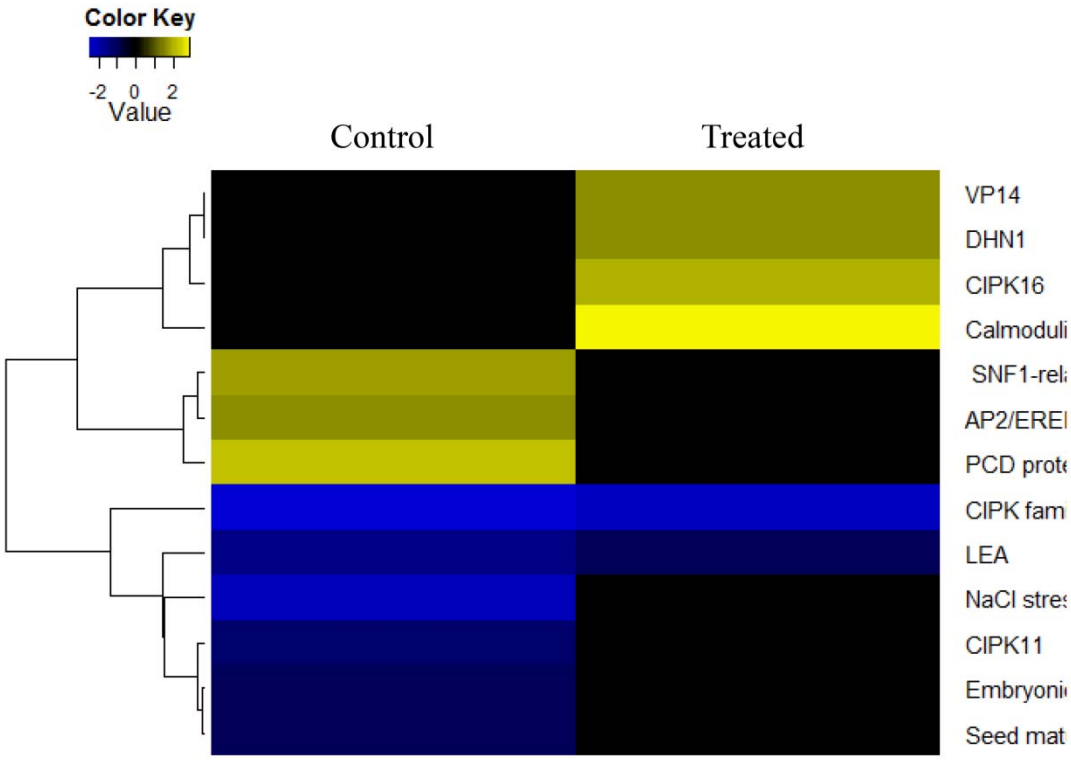



Figure 4.12: A heatmap representation of genes that are differentially expressed between wild type and *gcn2-1* in both control and PEG treated conditions.

indicated that GCN2 might play a role in endogenous ABA accumulation and ABA-mediated drought response in maize. Measurement of ABA abundance in maize leaves demonstrated that the endogenous ABA level was significantly higher in *gcn2-1* leaves compared to wild type. However, after PEG induced drought treatment, there were no significant differences in ABA abundance between wild type and *gcn2-1*. These data suggested that *gcn2* mutant seedlings were more tolerant to drought stress and that the increased tolerance may be related to the increased endogenous ABA abundance.

ABA biosynthesis is regulated by abiotic stresses at multiple steps. Both ABA-dependent and independent abiotic stress signaling first modify constitutively expressed transcription factors, leading to the expression of early response transcriptional activators, which then activate downstream stress tolerance effector genes (Zhu, 2002). Because of the complex regulatory mechanism and the lack of candidate genes to investigate why ABA abundance is elevated in *gcn2-1*, we performed an RNA-Seq analysis to look for differentially expressed genes between wild type and *gcn2-1* mutant and between control and treatment.

We found that there were much fewer gene expressions altered by PEG treatment in *gcn2-1* mutants, which was correlated with the reduced sensitivity of *gcn2-1* to drought and ABA accumulation. Candidate genes such as LEA, drought induced protein 1, CIPK-like protein, and wound/stress protein were substantially up regulated in wild type by PEG treatment, whereas no alteration was detected in *gcn2-1*. There were also candidate genes that were only up regulated in *gcn2-1* by PEG treatment, such as CIPK16, protein phosphatase 2C (PP2C) and several uncharacterized genes that were involved in various stress responses. PP2C has been reported to repress ABA signal transduction in normal

conditions, but stress-induced ABA binds to PYR/PYL/RCAR (PYL) receptors, which in turn binds and inhibits PP2C (Sheen, 1998; Takeuchi et al., 2014). The expression of *PP2C* is higher in *gcn2-1* after drought treatment compared to wild type, which could be caused by the increased baseline level of ABA in *gcn2-1*.

Further investigation is necessary to better understand why the steady state ABA abundance in *gcn2-1* leaves was significantly higher compared to wild type. By comparing wild type and *gcn2-1* gene expressions under control condition, we identified two differentially expressed candidate genes that are involved in ABA biosynthesis process. Beta-carotene hydroxylase is predicted to function in the biosynthesis of zeaxanthin, which is a carotenoid precursor of ABA. It is reported that overexpression of beta-carotene hydroxylase in rice and *Arabidopsis* significantly enhances stress tolerance, such as drought and oxidative stresses (Davison, et al., 2002; Du, et al., 2010). Although the expression level of beta-carotene hydroxylase is 6.5-fold lower in *gcn2-1* control leaves compared to wild type, the expression level increased drastically (42-fold) after PEG treatment and no differential expression was detected in treated leaves (Figure 4.11). Zeaxanthin epoxidase (ZEP, GRMZM2G127139) has been reported to be responsible for the conversion of zeaxanthin to violaxanthin in ABA biosynthesis (Audran et al., 2001). NCED functions after ZEP, by cleaving the major epoxycarotenoid 9-cis-neoxanthin to produce xanthoxin. Thompson et al. found that during drought stress, the expression of *NCED* transcript increased significantly in both tomato seedling roots and leaves, whereas *ZEP* transcript increase was only detected in roots (Thompson et al., 2000). *ZEP* was significantly down regulated in *gcn2-1* leaves compared to wild type in both control and treated conditions, which is not consistent with the elevated steady state ABA abundance

measured by GC-MS. Other genes known to be involved in ABA biosynthesis, such as ABA aldehyde oxidase (AAO), molybdopterin cofactor sulfurase (MCSU) and short-chain alcohol dehydrogenase/reductase (SDR) (Xiong and Zhu, 2003), were found to have similar expression levels in both genotypes.

The increased steady state ABA could be further investigated from the following aspects. Aside from *de novo* synthesis, ABA could also be transported from other tissues, such as roots (Schraut et al., 2004). Therefore, it might be feasible to investigate the expression levels of ABA synthesis genes in roots. In order to rapidly respond to ABA-mediated stresses, ABA could also be produced from deconjugation of its inactive form ABA glucosyl ester (ABA-GE) via catalyzation of ABA glucosyltransferase. In *Arabidopsis*, AtBG1 was reported to release ABA from ABA-GE, providing a quick adjustment of ABA abundance to allow plants to respond to the stressful conditions (Lee, et al., 2006). Thus the expression level of the maize AtBG1 homologue could also be investigated. In addition, genes involved in ABA degradation pathway could also be affected by the *gcn2* mutation.

The ABA inducible genes detected in the RNA-Seq analysis need to be validated by quantitative real-time-PCR (qRT-PCR). To validate the differentially expressed genes from RNA-Seq by qRT-PCR, candidates from both up and down regulated genes should be chosen. In addition, putative house-keeping genes that were relatively highly expressed and had low variations among samples can be identified to use as reference genes in qRT-PCR validation (O'Rourke). In addition, the level of eIF2α phosphorylation should also be tested in both genotypes after drought treatment. Studies in *Arabidopsis* reported that cold

stress induces eIF2α phosphorylation but it has not been determined whether drought could also increase the level of eIF2α phosphorylation (Lageix et al., 2008).

In addition, physiological analyses such as water use efficiency and stomatal conductance could be done to study the enhanced drought tolerance in *gcn2-1* leaves. Water use efficiency can be accurately measured by carbon isotope discrimination in C3 plants. It tests the ratio of the rate of the stable carbon isotope C-13 incorporation to the rate of transpiration during a certain period of time (Bowman et al., 1989). Although determining water use efficiency can be complex in C4 plants because of limited Rubisco discrimination, it has been used for selection of maize inbred and hybrid lines for drought tolerance (Monneveux et al., 2007). Plants of both genotypes could be treated with water withholding for 7 days and leaf tissues would be harvested and analyzed for their C-13 contents. Stomatal conductance analysis could indicate how fast guard cells sense the drought stress and close during drought treatment, a process considered to be regulated by ABA.

### *Authors' Contributions*

MJ Writing, experimental design and data (All figures and tables included). BCG Experimental design and writing.

### *Acknowledgement*

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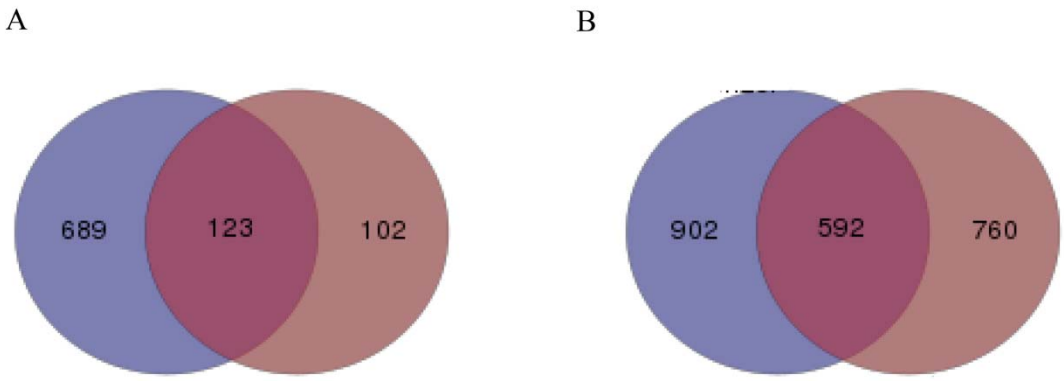
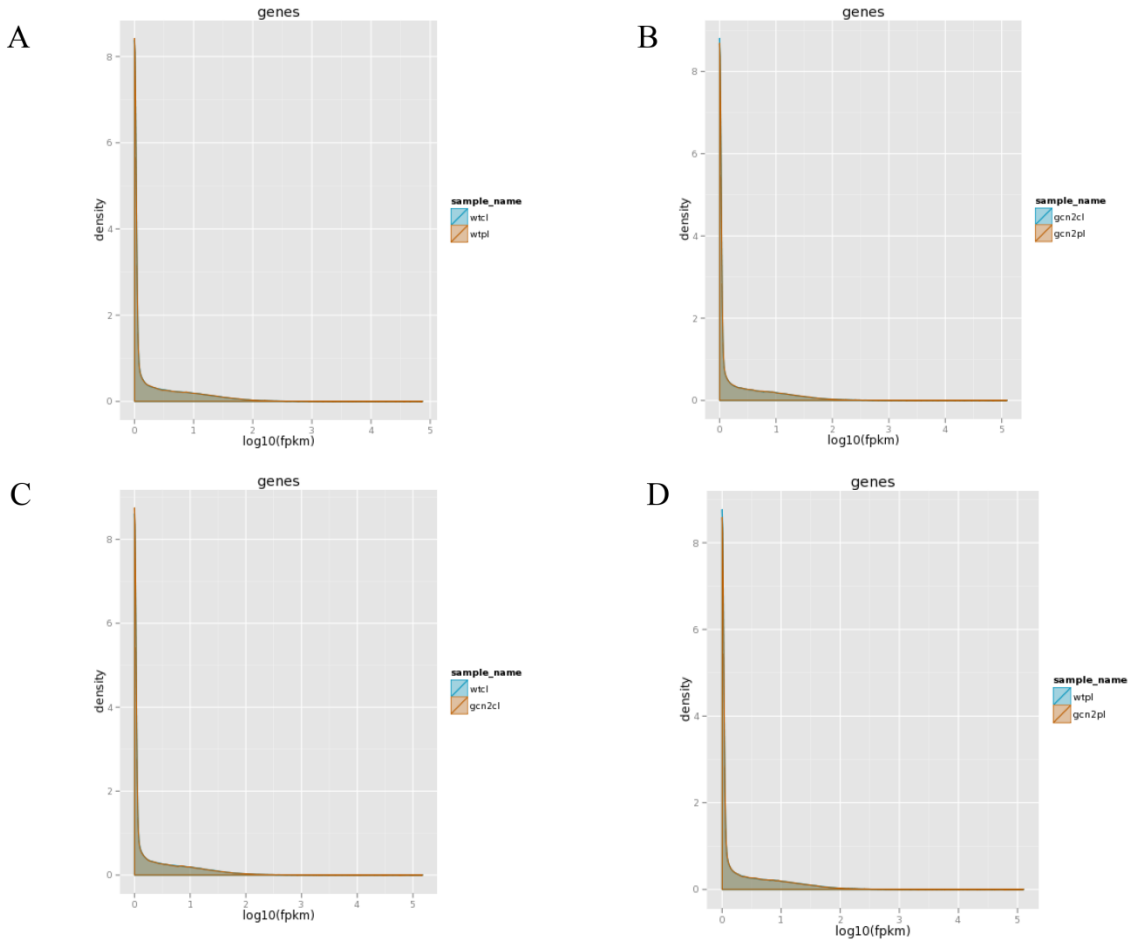
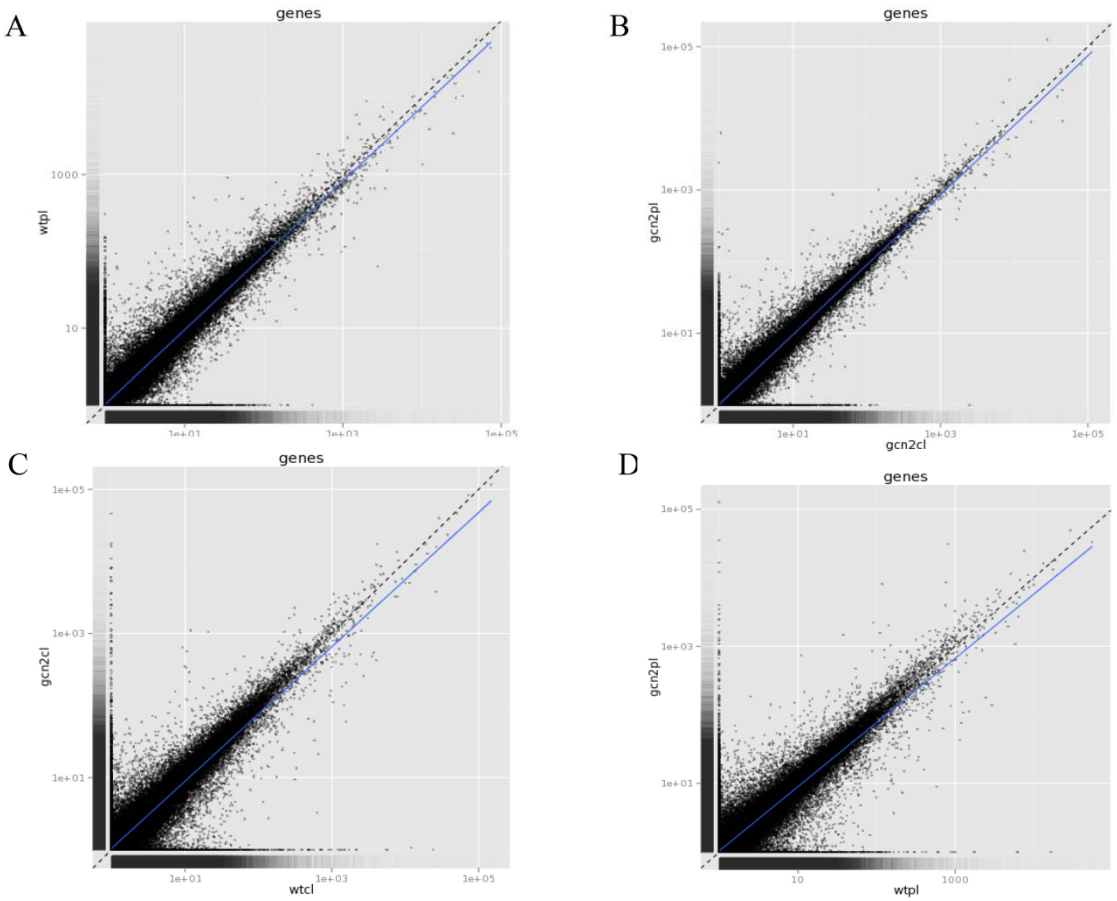


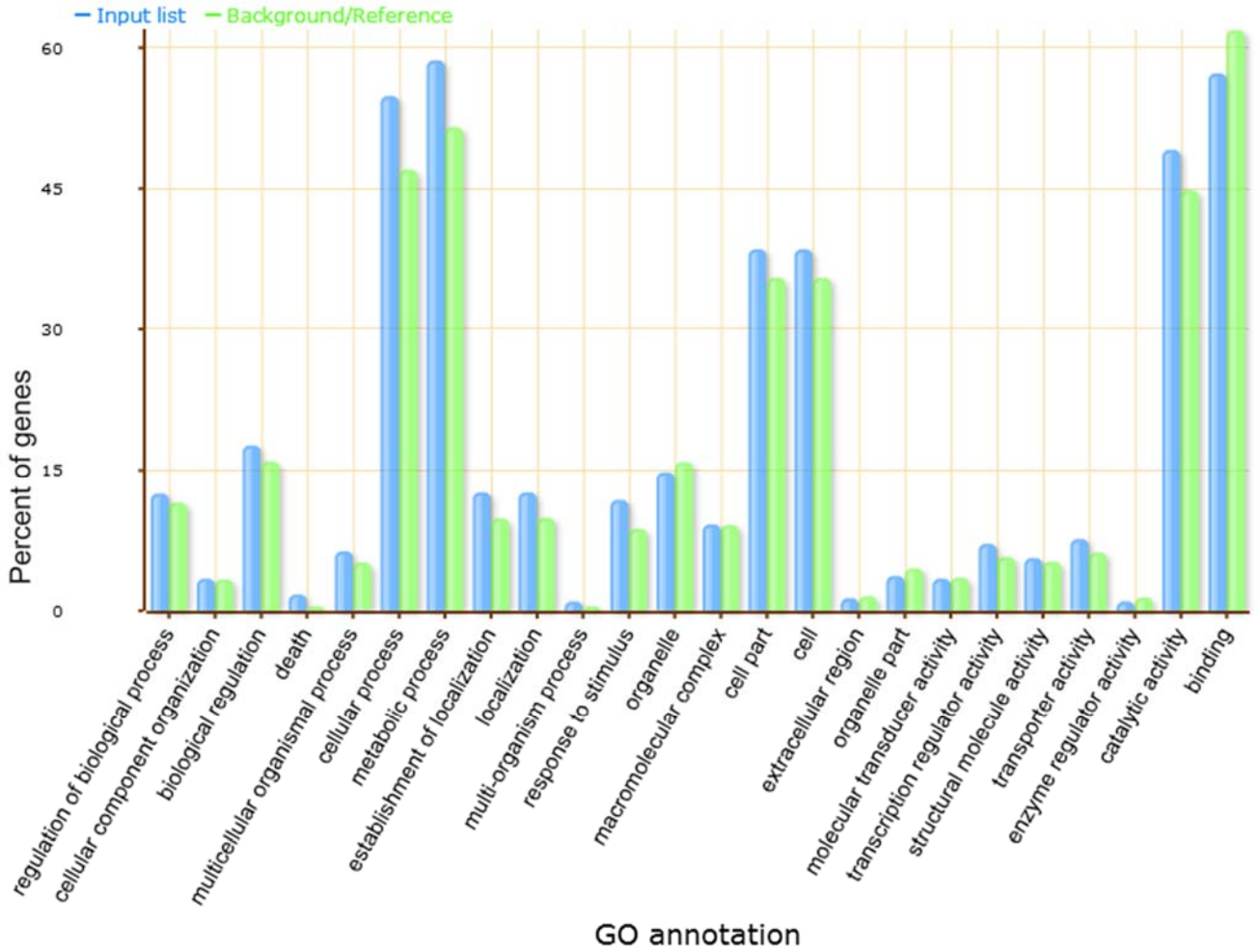

Supplemental Figure S4.1: Venn diagrams comparing shared gene expression between WT and *gcn2-1*. A) shared gene expression alteration between WT (left) and *gcn2-1* (right) after PEG treatment. B) shared gene expression alteration between control (left) and PEG treatment (right) by the two genotypes.



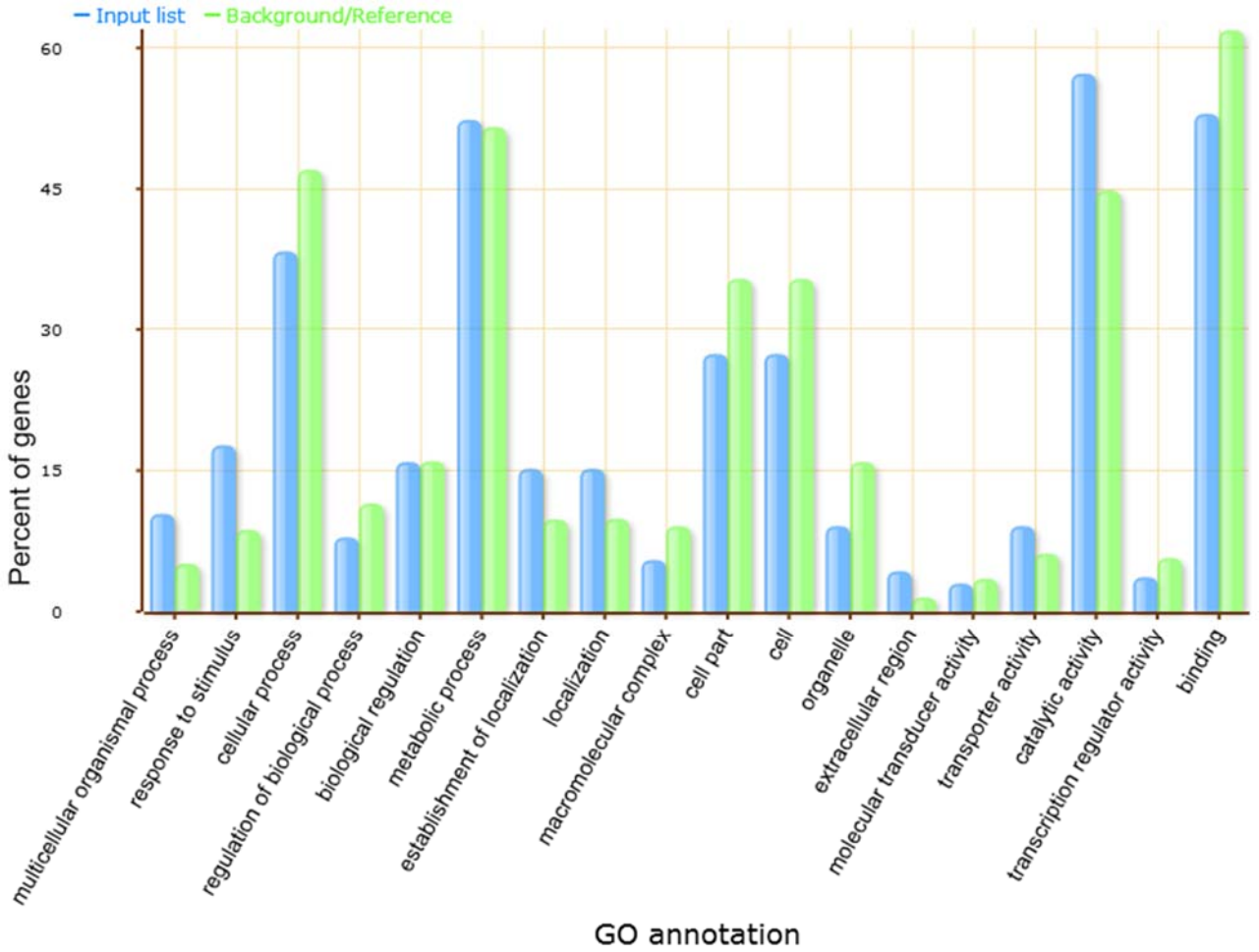

Supplemental Figure S4.2: Density plots of the expression level distributions for all genes in control and PEG treated conditions. A) wild type control and treated. B) *gcn2-1* control and treated. C) wild type control and *gcn2-1* control. D) wild type treated and *gcn2-1* treated.



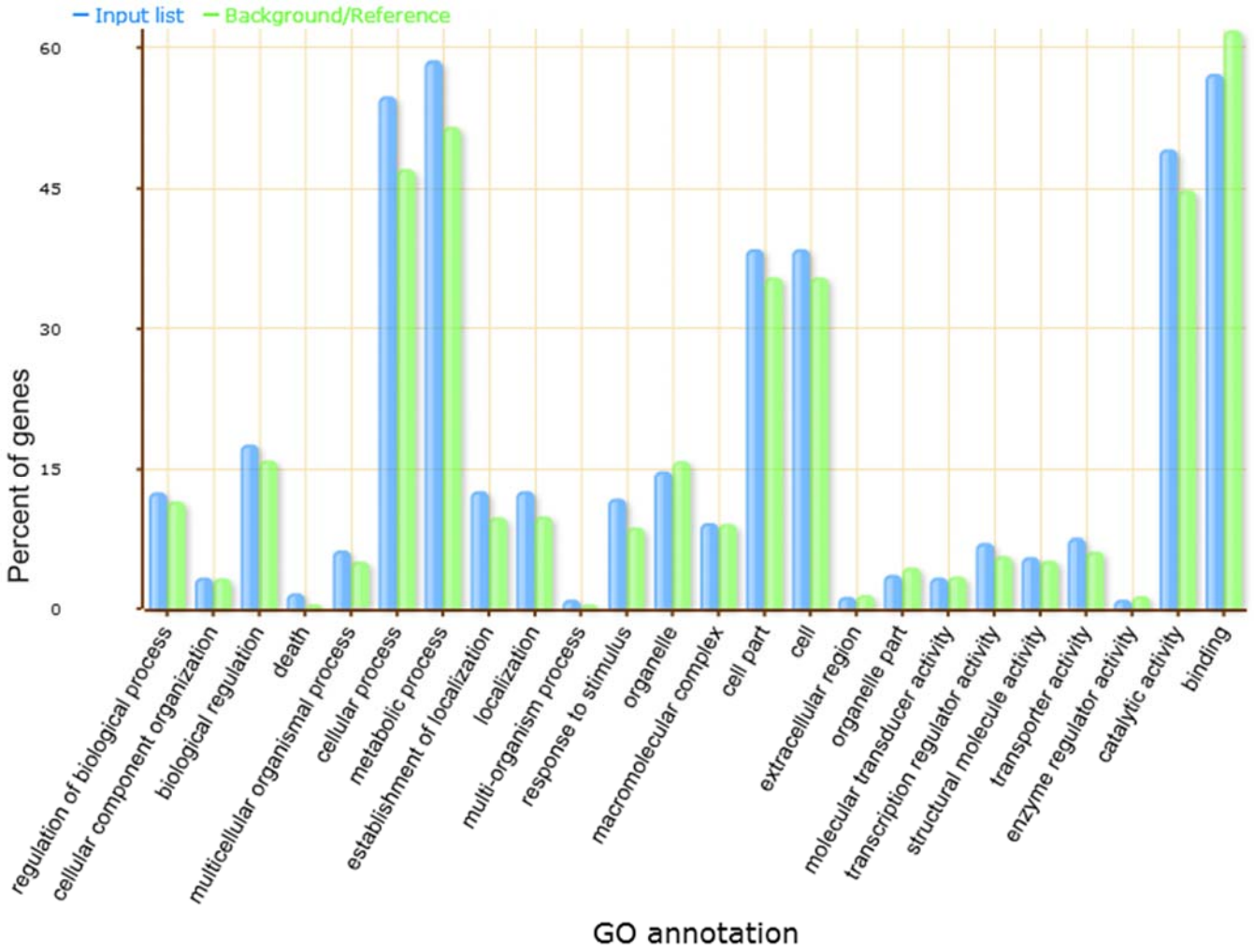

Supplemental Figure S4.3: Scatter plots of the expression level distributions for all genes in control and PEG treated conditions. A) wild type control and treated. B) *gcn2-1* control and treated. C) wild type control and *gcn2-1* control. D) wild type treated and *gcn2-1* treated.



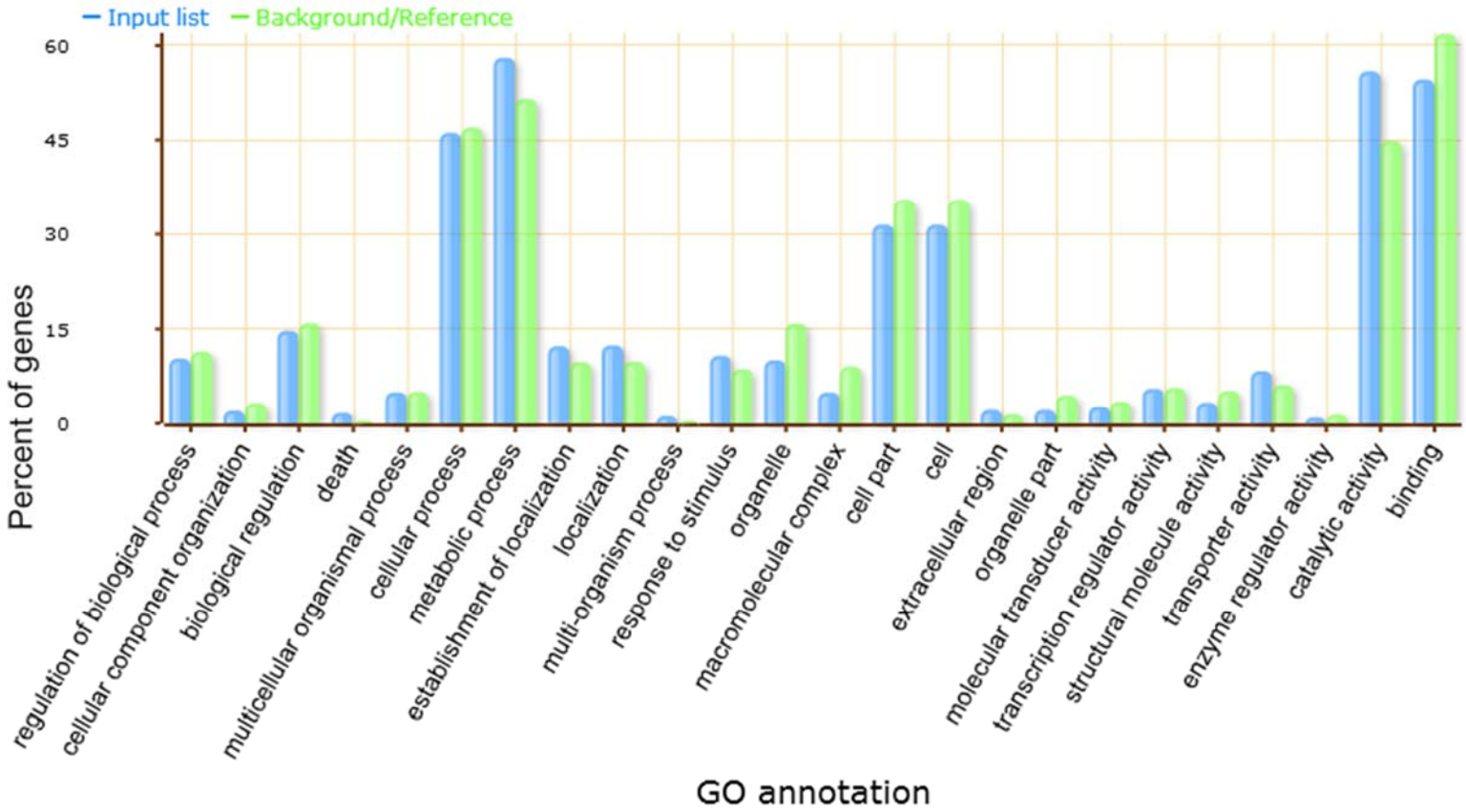

Supplemental Figure S4 4: Flash bar chart of over represented GO terms for drought induced candidate genes in wild type. The Y-axis is the percentage of the input genes mapped to different GO terms. The green bars represent the percentage of the GO terms in the reference genome.




Supplemental Figure S4 5: Flash bar chart of over represented GO terms for drought induced candidate genes in *gcn2-1*. The Y-axis is the percentage of the input genes mapped to different GO terms. The green bars represent the percentage of the GO terms in the reference genome.




Supplemental Figure S4.6: Flash bar chart of over represented GO terms for candidate genes altered by *gcn2* mutation in control condition. The Y-axis is the percentage of the input genes mapped to different GO terms. The green bars represent the percentage of the GO terms in the reference genome.




Supplemental Figure S4.7: Flash bar chart of over represented GO terms for candidate genes altered by *gcn2* mutation in drought condition. The Y-axis is the percentage of the input genes mapped to different GO terms. The green bars represent the percentage of the GO terms in the reference genome.

# CHAPTER FIVE

## Maize Translation Initiation Factor 5A is Associated with a Cytoskeleton Rich Fraction in Maize Endosperm


*Abstract*

The formation of prolamin storage protein bodies in the endoplasmic reticulum is a highly regulated process that results in the ordered deposition of several classes of proteins in specific regions of the protein body. Even small perturbations in the arrangement of the different classes of proteins result in a stress response in the ER and can result in an opaque phenotype of maize endosperm. One of the earliest events in protein body formation is the deposition of cysteine rich glutelin proteins such as the 27 kDa γ-zein. Experiments in rice suggest that the mRNA for such proteins accumulate in discrete regions of the ER around forming protein bodies. We performed gel mobility shift assays to identify endosperm proteins that could bind to a probe comprising the 3' untranslated region of the maize 27 kDa γ-zein mRNA. Among the proteins bound to the probe we identified eIF5A, a unique translation factor that contains the unusual amino acid hypusine and is often upregulated under stress conditions in plants. Proteomic analysis of co-precipitating proteins revealed that eIF5A is associated with a cytoskeleton rich fraction that includes actin, tubulin, EF1A and ribosomal proteins. Pairwise immunoprecipitation experiments showed that eIF5A could bind directly to both actin and EF1A, whereas the association with tubulin may be dependent on its interaction with EF1A. These associations were not dependent on the post-translational modification of lysine 53 to hypusine.

Association of eIF5A with ribosomes was dependent on the presence of hypusine, consistent with prior reports. These data suggest that eIF5A may play a role in the targeting of glutelin precursor mRNAs to the endoplasmic reticulum.

## *Introduction*

Seed storage proteins play a key role in plant growth and development. They provide nutrition and energy for seeds during germination and the early growth of the seedlings. In maize endosperm, prolamin storage proteins (zeins) are the major storage proteins. There are several groups of zeins found in maize endosperm, named α-, β-, γ-, and δ-zeins. These zeins accumulate as spherical accretions called protein bodies (PB), and they are synthesized on the rough endoplasmic reticulum (ER) and are retained in the lumen of the ER (reviewed by Boston and Larkins, 2009; Herman and Larkins, 1999; Holding and Larkins, 2006). The assembly of zeins within the ER is a highly regulated process in which β- and γ-zeins are synthesized first throughout the PB during the early stage of PB development and then α- and δ-zeins are synthesized inside the matrix of β- and γ-zeins, expanding the size of PB to a diameter of 1 to 2 μm. The mature PB structure contains α- and δ-zeins at the center and β- and γ-zeins at the peripheral region (Herman and Larkins, 1999; Lending et al., 1989; Shewry and Halford, 2002).

Studies have found that γ-zein regulates at least the organization of α-zein and contributes to the retention of α-zein within the ER lumen after synthesis, because α-zein will only accumulate into complexes with the presence of γ-zein in vivo when synthesized simultaneously in transgenic tobacco (Coleman et al., 1996). The proline-rich repetitive sequences at the N terminus of γ-zein were found to be required for its ER retention (Geli et al., 1994), and the specific formation of zeins into mature PB could be accomplished

through potential protein-protein interactions based on the distinct structure of each zein group (Coleman et al., 1996; Holding and Larkins, 2006).

Since γ-zein is very important for the correct formation and localization of PB, it is necessary to investigate how γ-zein is synthesized and retained in specific regions of the ER. Li et al found that prolamin mRNAs were enriched on the rough ER surrounding developing PB in rice endosperm (Li et al., 1993). Furthermore, Muench et al reported that the targeting of mRNAs to the rough ER could be carried out by interactions with cytoskeleton associated proteins such as tubulin and actin (Muench et al., 1998). It is also reported that transcript specific binding proteins could interact with 3' untranslated region (UTR) of the mRNAs and direct the localization of the mRNAs and subsequent protein synthesis (St. Johnston, 1995). It is not clear that a similar mechanism operates in maize endosperm because the distribution of γ-zein mRNA on the ER was not enriched on membranes surrounding PB. However, the hybridization of the probes was observed in clusters and enriched on ER membranes. Based on these observations, we hypothesize that the γ-zein is discretely localized to the rough ER through an mRNA-sorting mechanism where the γ-zein mRNAs are targeted to the ER by transcript specific binding proteins that bind to the 3' UTR and also interact with the cytoskeleton.

In this study, proteins capable of binding to a 3' UTR probe from the 27 kDa γ -zein were identified by gel mobility shift assays. One of the protein fractions that contained an 18 kDa protein was identified as maize eukaryotic translation initiation factor 5A (ZmIF5A). The *ZmIF5A* gene was expressed in all tissues examined and was particularly abundant in tissues with high rates of cell division and protein synthesis. Furthermore, ZmIF5A was found to be associated with a cytoskeleton rich fraction and this interaction

is likely to be mediated by binding to EF1A and F-actin. Notably, the association of eIF5A with F-actin was hypusine dependent. ZmIF5A was also found to bind to ribosomes in a hypusine-dependent manner. These results suggest that ZmIF5A could contribute to the localization of γ-zein mRNAs to the rough ER by promoting associations with both the cytoskeleton and translational apparatus.

## *Materials and Methods*

### *ZmIF5A Isoform Identification*

ZmIF5A isoforms were identified by a BLAST search in http://maizesequence.org with the full length sequence of isoform 1 (MaizeGDB Accession no. GRMZM2G144030) and confirmed by the existence of predicted functional domains in their protein sequences. Gene specific primers were designed for qRT-PCR to recognize the 3' UTR region of each isoform. Multiple alignment of ZmIF5A protein sequences was performed using the Geneious alignment algorithm in Geneious (Biomatters, Aukland, NZ).

### *Expression Analysis of ZmIF5A in Plant Tissues*

Kernels of B73 inbred line for qRT-PCR and western blotting were grown and harvested in Elm Mott, TX during the summer of 2012. For ZmIF5A expression analysis, kernels of different developmental stages were harvested (10, 14, 18, 20 and 22 DAP) and kept frozen at -80 °C; seedlings were grown till 14-day-old and then harvested, dissected into leaves and roots, frozen in liquid nitrogen and stored at -80 °C. For endosperm materials, three ears of each developmental stage were used as three biological replicates, with six endosperms dissected and pooled as one replicate. For vegetative tissues, leaves or roots from three 14-day-old seedlings were pooled as one replicate and three biological

replicates were used for the analysis. Maize inbred line W64A+ 18 DAP endosperm was used for total protein extraction in the recombinant eIF5A pull-down assay.

Total RNA was extracted from plant materials described above (dissected 10, 14, 18 and 22 DAP endosperms, 14-day-old seedling roots and leaves) with Plant RNA Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. One μg of total RNA was used for first-strand cDNA synthesis using qScriptTM cDNA SuperMix (Quanta Biosciences, Gaithersburg, MD) and was diluted 10-fold prior to use in qRT-PCR.

*Quantitative Real-Time PCR Analysis of ZmIf5A Isoforms*

qRT-PCR was performed using PerfeCTa® SYBR® Green FastMix® (Quanta Biosciences, Gaithersburg, MD) in Corbett Rotor-GeneTM 3000 (Qiagen, Valencia, CA). Plasmid solutions with copy numbers ranging from 103 to 107 were used to create the standard curve. The PCR program was as follows: 50°C hold for 2 min for auto gain optimization, 95°C initial denaturing for 10 min, 50 cycles of 95°C for 15 s and 60°C for 1 min. Expression levels of eIF5A isoforms were normalized against the expression of the retinoblastoma-related protein 1 (RRB1; Grafi et al., 1996) in the corresponding samples. High resolution melting curves were obtained to verify the presence of single amplicons. Data were analyzed using the Q-Gene Core Module file (Simon, 2003). Statistical differences among the isoforms were evaluated by unpaired two-tailed student's t-test.

*Protein Extraction, SDS-PAGE and Western Blotting*

As described above, pooled maize B73 endosperms were ground into a fine powder in liquid nitrogen. Total protein was extracted from 50 mg of the ground powder mixed with borate buffer (12.5 mM sodium borate, 1% (w/v) SDS, 2% β-mercaptoethanol, pH

10) (Wallace et al., 1990). After incubation at room temperature for 2 hrs, samples were centrifuged and the supernatant was collected. The zein proteins were then removed from the total extract by precipitating the non-zein proteins with 70% ethanol and resuspended the non-zein precipitates in 1X Laemmli sample buffer.

Maize seedling leaf or root tissues were ground into fine powders in liquid nitrogen and total proteins were extracted using P-PER Plant Protein Extraction Reagent (Thermo Fisher Scientific, Rockford, IL) according to manufacturer's instructions. Protein concentrations were determined by reducing agent compatible Bradford assay. Equal volumes of non-zein fractions (~10μL) were heated at 95°C for 10 min and then separated on a 12.5% SDS-PAGE gel. Twenty-five μg of total proteins from leaf or root tissues were separated on a 12.5% SDS-PAGE gel. Proteins were then transferred to a Protran nitrocellulose membrane (Whatman, Pensacola, FL) and western blotting was performed with the custom rabbit anti-ZmIF5A as the primary antibody at a 1:3000 dilution and a HRP-conjugated goat anti-rabbit antibody at a 1:30,000 dilution (Invitrogen, Carlsbad, CA). The presence of ZmIF5A was detected using ImageQuant LAS 4000 imaging system (GE Healthcare, Piscataway, NJ) after treating the membrane with Amersham ECL western blotting detection reagents (GE Healthcare, Piscataway, NJ). The band intensity was quantified using the ImageJ software (Schneider et al., 2012) and the statistical differences were evaluated by unpaired two-tailed student's t-test.

*Purification of Native ZmIF5A*

Native ZmIF5A was purified from ~50g of frozen maize kernels. Maize endosperm proteins were fractionated by precipitation between 40-80% saturated ammonium sulfate. The dialyzed pellet was passed over a Q-Sepharose column and eluted with a gradient of

0-500 mM NaCl. The fractions containing eIF5A protein were dialyzed and passed over a Phenyl-Sepharose column and eluted with a gradient of 2M-0 ammonium sulfate. Finally, the fractions containing eIF5A were dialyzed and passed over a Uno-Q1 column (Bio-Rad, Hercules, CA) and eluted with a gradient of 0-500mM NaCl. The fractions were separated on a SDS-PAGE gel and the ones that contained pure and highly concentrated native ZmIF5A protein was aliquoted and stored at -80°C.

*Protein-Protein Interaction of ZmIF5A and Target Proteins*

Ten μM native and recombinant ZmIF5A was covalently linked to NHS Mag Sepharose beads according to manufacturer's instruction (GE Healthcare, Piscataway, NJ). Purified target protein (5μM) in protein binding buffer was added to each reaction and incubated with end-to-end mixing. Purified rabbit skeletal muscle actin (Cat. No. AKL95, Cytoskeleton, Denver, CO) was used for interactions of globular and filament actin. Globular actin was prepared by adding 100μL water to the lyophilized protein and incubated on ice for 1 hr to depolymerize actin oligomers. Filament actin was prepared by adding polymerization buffer (50mM KCl, 20mM $MgCl_2$, 10mM ATP) to globular actin and incubate at RT for 1 hr. Native EF1A protein was purified by a similar method of purification of native ZmIF5A protein, except that the first column used was SP-Sepharose instead of Q-Sepharose. Recombinant eIF2α was provided by Karen Browning. NHS beads not linked to any protein were used as a negative control for nonspecific binding and trapping. After incubation, the beads were washed three times with HNTG buffer (20mM HEPES pH 7.5, with 150 mM NaCl, 0.1% (w/v) Triton X-100, and 10% (w/v) glycerol) and proteins bound to ZmIF5A proteins were eluted with glycine elution buffer and identified on SDS-PAGE gels followed by western blots.

*ZmIF5A Transcript Expression Analysis*

To further characterize ZmIF5A, a BLAST search using ZmIF5A protein sequence acquired from NCBI (CAA69225.1) was performed against the maize B73 genome. Three different genes of ZmIF5A were identified based on sequence similarity and conserved functional domains, with isoform 1 located on chromosome 7 and isoforms 2 and 3 located on chromosome 2, separated by 26 Mb (GRMZM2G144030, GRMZM2G113696 and GRMZM2G082974, respectively). Multiple alignment showed that isoforms 1 and 2 share

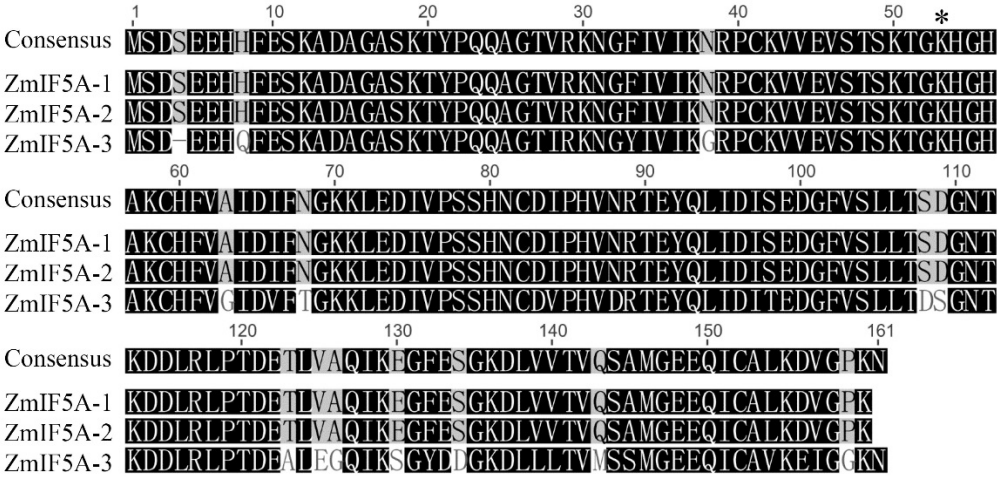



Figure 5.1: Protein sequence alignment of ZmIF5A isoforms. Isoform 1 and 2 share the same amino acid sequence but isoform 3 is less well conserved (82.5% identity). The asterisk indicates the conserved lysine that becomes hypusinated.

the same amino acid sequence, whereas isoform 3 is less well conserved, sharing 82.5% identity with the two (Figure 5.1). Because the nucleotide sequences of the three isoforms share less similarity (less than 70%), isoform specific primers were designed for transcript expression analysis (Supplemental Table S5.1).

To investigate the transcript expression levels of three ZmIF5A genes, quantitative real-time PCR (qRT-PCR) was performed using developing endosperms, root and leaf tissues from 14-day-old seedlings. As shown in Figure 5.2, the expression of all three *ZmIF5A* isoforms was detected in the tested tissues. Isoforms 1 and 2 were shown to have similar expression levels, whereas isoform 3 was expressed at a significantly lower level (For developing endosperms, $p < 0.001$, n=3; for root and leaf tissues, $p < 0.05$, n=3). This could be due to the distinct biological function of ZmIF5A isoform 3, as described for Arabidopsis (Thompson et al., 2004). Transcript levels of the three isoforms appeared to decrease as maize endosperm develops, with the highest expression level at 10 DAP and lowest level at 22 DAP (Figure 5.2A). The three isoforms are expressed at a significantly higher level in seedling roots compared to leaves (Figure 5.2B; $p < 0.05$, n=3).

*ZmIF5A Protein Abundance Analysis*

To analyze the abundance of total ZmIF5A, an antiserum was raised against recombinant 6X His-tagged ZmIF5A isoform 1. Maize endosperm tissues dissected from 10, 14, 18, 20, and 22 DAP were fractionated to remove the zein proteins, separated by SDS-PAGE and then analyzed by western blot using the anti-ZmIF5A antibody with an anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody as a loading control (Figure 5.3A). The developing endosperms were extracted with the same ratio of material weight over extraction buffer so that equal volume of samples would contain equal amounts of total protein. The total non-zein protein abundance was low at the 10 DAP stage, but stayed relatively constant in the later stages (14-22 DAP). Western blot analysis showed that during maize endosperm development, the abundance of ZmIF5A increased from 10 DAP to 14 DAP, peaked at 14 DAP, and decreased as endosperm continued to develop, with the

lowest abundance detected at 22 DAP. The changes of ZmIF5A protein abundance were not consistent with the trends of ZmIF5A transcript expression, as there was a delay of change in protein abundance during endosperm development. However, the abundance of GAPDH showed a gradual decrease, with the highest accumulation detected at 10 DAP and lowest at 22 DAP.

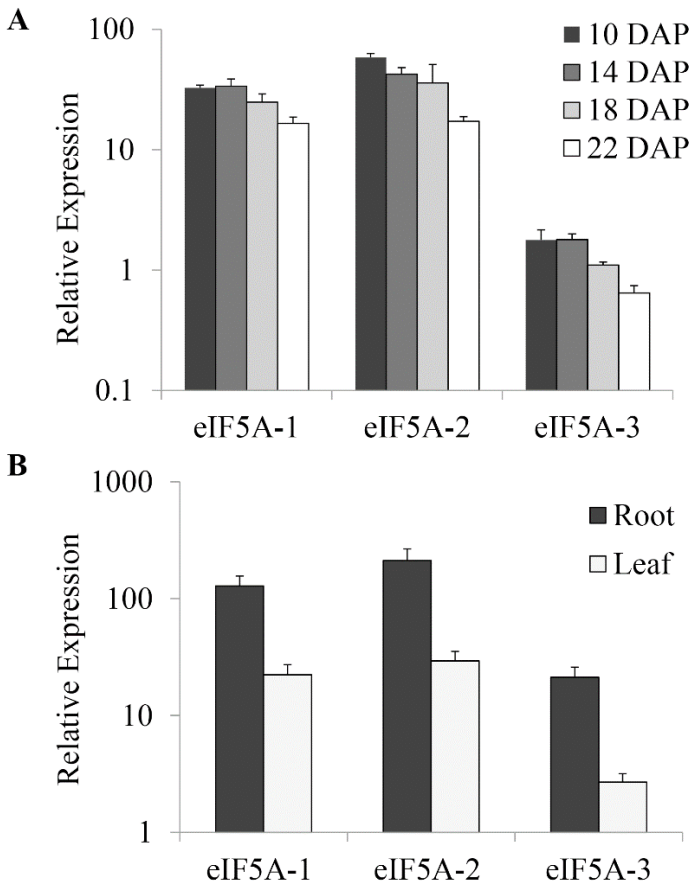



Figure 5.2: Quantitative RT-PCR analysis of *ZmIF5A* isoforms in different maize tissues. A) Expression of *ZmIF5A* isoforms in developing endosperms. The transcript expression level of all three isoforms appears to decrease as endosperm develops. B) Expression of *ZmIF5A* isoforms in vegetative tissues (14-day-old seedlings). All three isoforms are expressed significantly higher in roots compared to leaves ($p<0.05$). Expression was normalized to maize RRB1. *ZmIF5A* isoforms 1 and 2 are expressed at a similar level in tested tissues, whereas *ZmIF5A* isoform 3 is expressed 10 fold lower ($p<0.05$).

The abundance of ZmIF5A in maize seedling root tips and leaves were also analyzed by western blot. As shown in Figure 5.3B, ZmIF5A accumulation was significantly higher in maize seedling root tips compared to leaves (Table 5.1; $p < 0.01$, n=3). Densitometry analysis was done with the integrated densities of ZmIF5A normalized against that of GAPDH to correct for protein loading and minimize factors such as uneven transfer efficiency. The difference in ZmIF5A abundance between seedling roots and leaves were consistent with the difference detected at the transcript level (Figure 5.2B).

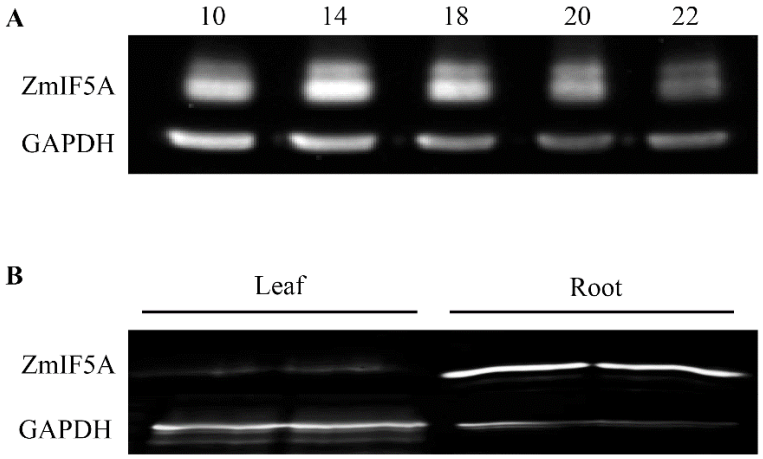



Figure 5.3: Western blot analysis of ZmIF5A abundance in different maize tissues. A) Western blots with antibodies against ZmIF5A and GAPDH. Non-zein proteins from dissected endosperm at 10, 14, 18, 20, and 22 DAP were analyzed. Protein accumulation increased at 14 DAP but decreased later in development, showing a delay of change to transcript abundance. GAPDH was used as a positive control to indicate the general decrease of non-zein proteins during endosperm development. B) Western blot analysis of eIF5A abundance in B73 vegetative tissues.

Table 5.1: Densitometry analysis of western blots of eIF5A abundance in 14-day-old maize seedlings (n=3).

| | Root | | Leaf | | |
|---|---|---|---|---|---|
| Protein | Mean | SD | Mean | SD | p |
| ZmIF5A | 6.64 | 0.48 | 4.08 | 0.32 | |
| GAPDH | 1.99 | 0.13 | 2.58 | 0.27 | |
| Normalized Value | 3.34 | 0.22 | 1.59 | 0.08 | < 0.01 |

*Confirmation of Direct Protein Interactions with ZmIF5A*

To confirm the protein interactions with ZmIF5A detected above, recombinant and native ZmIF5A proteins were coupled separately to NHS Mag Sepharose beads and incubated with purified target proteins. The proteins used to confirm the interactions were recombinant eIF2α, native globular and filamentous actin (G- and F-actin, respectively), and native EF1A. The beads were washed after incubation and the proteins bound to the beads were eluted and identified by SDS-PAGE followed by western blot analysis. Uncoupled beads were used as a negative control for trapping. Both native and recombinant ZmIF5A proteins were found to interact with eIF2α, G-actin and EF1A. The interaction of EF1A was stronger with recombinant ZmIF5A compared to native ZmIF5A, whereas the interaction of G-actin was stronger with native ZmIF5A. The association with these proteins appears to be hypusine independent. However, F-actin was found to only interact with native eIF5A (Figure 5.4). These results confirmed some of the protein interactions identified in the pull-down assay and immunoprecipitation. The stronger signal of actin in the immunoprecipitation elution could be both G- and F-actin, as confirmed above. EF1A was previously reported to be associated with F-actin in the cytoskeletal network in

endosperm and the association may a role in PB deposition (Clore et al, 1996). Therefore, the interaction of ZmIF5A with both F-actin and EF1A will provide insight into possible cellular roles of ZmIF5A, such as its function in the localization of γ-zein mRNAs.

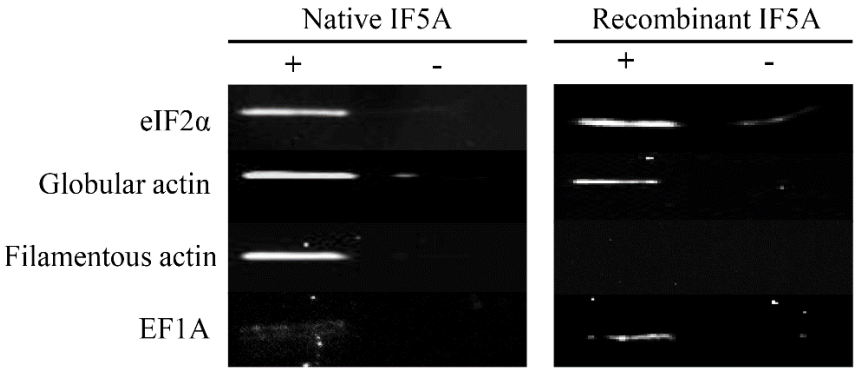



Figure 5 4: Confirmation of direct interaction between ZmIF5A and purified target proteins. Native and recombinant ZmIF5A were purified and covalently linked to NHS magnetic beads. Purified target protein (5μM) was added to each reaction and incubated with end-to-end mixing. NHS beads were washed after incubation and proteins bound to the beads were eluted and identified on SDS-PAGE gels followed by western blots. Both native and recombinant ZmIF5A proteins were shown to interact with eIF2α, G-actin and EF1A. The association with these proteins appears to be hypusine independent. However, only native ZmIF5A was shown to interact with F-actin.

*Discussion*

The essential protein eIF5A has been found to play a role in diverse cellular processes. It is the only protein that contains the unusual amino acid hypusine. Although early experiments suggested that eIF5A had a role in the initiation step of translation, depletion studies with yeast from numerous laboratories have proved otherwise (Kang and Hershey, 1994; Klier et al., 1995). Recently it has been proposed that eIF5A plays a role in the elongation step of translation. In vitro assays with yeast eIF5A mutants demonstrated that upon addition of hypusine modified wild type eIF5A, but not the mutated K51R strain, a marked increase in translation of luciferase reporter mRNAs occurred (Saini et al., 2009).

Through yeast two-hybrid screens and co-purification experiments, eIF5A was found to associate preferentially with actively translating ribosomes and also with elongation factor EF2. The associations with ribosomes and EF2 are clearly dependent on hypusine modification of eIF5A (Zanelli et al., 2006). It was also demonstrated that temperature sensitive eIF5A yeast mutants caused an increase of the ratio between polysomes and monosomes, leading to the suggestion that while eIF5A has a modest effect on cell growth and translation under normal conditions, it has strong effects on cell proliferation under times of great environmental stress (Li et al., 2010). Under stress conditions, eIF5A knockdown cells have severely inhibited stress granule assembly, and reduced rates of ribosomal transit time in cultured mammalian cells (Li et al., 2010). These results indicate that eIF5A could be a non-essential elongation factor that is necessary when other elongation factors are inactiveated due to stress, and may be a critical factor for whether a cell survives unfavorable conditions.

The role of eIF5A in plants is not well studied, but the overall role to influence cell proliferation and responses to stress appears to be conserved. Maize infected with the facultative pathogen Curvularia lunata exhibited increased levels of eIF5A (Huang et al., 2009a; Huang et al., 2009b). One general feature of eIF5A expression in plants is that it tends to be more highly expressed in senescing tissues or in times of heat or drought stress (Thompson et al., 2004). In fact, overexpression of eIF5A in Arabidopsis enhanced thermotolerance, oxidative stress resistance and osmotic stress tolerance (Xu et al., 2011). The protein is also highly accumulated in the sperm cells of maize pollen (Dresselhaus et al., 1999) and in the phloem of pumpkin and Arabidopsis, where it might be involved in regulating translation of messages in the enucleate cells (Ma et al., 2010a; Ma et al., 2010b).

Transport into the phloem was shown to be independent of hypusine, but association with translation factors and ribosomes was hypusine dependent as previously found for yeast (Ma et al., 2010a).

In this study, using 3' UTR of the 27 kDa γ-zein mRNA as a probe, we identified ZmIF5A as one of the γ-zein mRNA binding proteins. The competition binding assay indicated that the binding of ZmIF5A to γ-zein mRNA was specific, because neither random RNA polymers nor poly-A homopolymer significantly altered the mobility of the probe. To investigate whether this association indicated a possible role of ZmIF5A in the localization of γ-zein synthesis and retention, ZmIF5A was further characterized to study its abundance in various tissues of maize and to identify its interacting proteins.

Three isoforms of ZmIF5A were identified in maize by BLAST analysis. These isoforms were named ZmIF5A-1, -2, and -3, in the order of the deposition times into the Genbank. Most eukaryotic organisms have two isoforms of eIF5A, while Arabidopsis and maize have three. In maize, it is shown that while all three isoforms are present in examined maize tissues, *ZmIF5A-1* and *-2* are expressed at significantly higher levels than the *ZmIF5A-3*.

The importance of ZmIF5A in cellular proliferation has been established using many eukaryotic organisms, but little work has been done with plants (Chen and Chen, 1997; Frigieri et al., 2007; Nishimura et al., 2005; Park, 2006; Schnier et al., 1991). Using maize tissues known to have high levels of proliferation or translational activity, such as endosperm and root tips, the expression of ZmIF5A was examined at both transcript and protein levels. During maize endosperm development, the cells undergo rapid mitosis and cell proliferation at the early stage (10 DAP) and the level of mitotic activity decreases as

endosperm continues to develop. At 20 and 22 DAP stages, the endosperm goes through programmed cell death and the cell proliferation level becomes low (Olsen, 2001). The decrease of all three ZmIF5A isoforms could indicate the role of ZmIF5A in cell proliferation during early stages of endosperm development. However, it is known that as endosperm develops, zein mRNAs take up an increasing percentage of the mRNA pool, reducing the apparent levels of many non-zein transcripts. ZmIF5A protein expression levels showed similar trends as its transcript levels in different tissues. The only inconsistency detected was that the ZmIF5A protein accumulation level peaked at 14 DAP, whereas its transcript had the highest expression level at 10 DAP. Because root tips contain the apical meristem that has high mitotic activity, ZmIF5A expression is expected to be high if it plays a role in cell proliferation in plants as has been observed in animals and fungi. Consistent with this hypothesis, all three ZmIF5A isoforms showed higher expression levels as shown in Figure 5.4B. In contrast, *ZmIF5A* transcript levels are on average seven-fold lower in leaves.

ZmIF5A associated proteins were investigated through pull-down analysis and immunoprecipitation. Pull-down analysis utilized recombinant ZmIF5A produced from *E.coli* cells and thus was not hypusinated. Among the proteins that were identified to interact with recombinant ZmIF5A, there were proteins related to the cytoskeleton, such as actin, the plastid tubulin homolog FtsZ, and three types of tubulin. Multiple translation elongation factors such as eukaryotic translation elongation factor 1 alpha (EF1A), translation elongation factor 1 gamma-3, and translation elongation factor thermo unstable, mitochondrial EF-TuM were also identified. In addition, there were metabolic enzymes identified, such as 1,4- α-glucan-branching enzyme 2, sorbitol dehydrogenase homolog1,

UDP-glycosyltransferase, alanine transaminase, alanine aminotransferase, acetolactate synthase, and pyruvate phosphate dikinase 1. An ATP-dependent 26S proteasome regulatory subunit was also identified [Saladine Master's Thesis, 2012]. To investigate whether the ZmIF5A interacting proteins were hypusine dependent, their association with native ZmIF5A were also determined by immunoprecipitation. Interestingly, ZmIF5A was found to interact with recombinant eIF2α, and this interaction was hypusine independent. eIF2α plays an important role in regulating global protein translation rate when cells are under stress (Hinnebusch, 2005). In response to certain stresses, such as amino acid starvation or UV radiation, eIF2α will be phosphorylated and causes a decrease in protein translation rate (Lageix et al., 2008; Zhang et al., 2008). It would be interesting to study whether ZmIF5A is also associated with phosphorylated eIF2α since maize endosperm is a hypoxic environment (Rolletschek et al., 2005). On the contrary, eIF4G was found to have no interaction with ZmIF5A, in both cases. Tubulin was not detected by immunoprecipitation. Its interaction with recombinant ZmIF5A may be an indirect interaction due to the binding of EF1A, which is a known microtubule binding protein (Durso and Cyr, 1994). RPS6, on the other hand, was detected only by immunoprecipitation, suggesting that the interaction between ribosomes and ZmIF5A is hypusine dependent.

Individual protein-protein interaction experiment further clarified whether the ZmIF5A binding proteins detected were directly interacting with ZmIF5A or they belonged to a complex and were pulled down or precipitated because of other proteins in the complex. Moreover, to distinguish whether the association with actin indeed indicated the association with the cytoskeleton, both G-actin and F-actin were tested for their interaction

with ZmIF5A. G-actin was shown to co-precipitate with ZmIF5A, and the association was hypusine independent. However, F-actin appeared to be only associated with native ZmIF5A, which indicated that the association with the actin cytoskeleton is hypusine dependent. It also explained the stronger signal of actin detected by immunoprecipitation.

### *Authors' Contributions*

MJ Writing, experimental design and data (Figures 5.1 to 5.4, Table 5.1 and Supplemental Table S5.1). SJS Writing, experimental design and data (ZmIF5A pulldown assay, protein identification and immunoprecipitation; Figures 6 and 7, and Table 2 in the manuscript, not included in this dissertation). BCG Writing, experimental design and data (Identification of ZmIF5A as a γ-zein mRNA 3' UTR binding protein and the competitive gel mobility shift assay; Figures 1 and 2 in the manuscript, not included in this dissertation). All authors read and approved the final manuscript.

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*Supplemental Data*

| Primer Name | Sequence |
|---|---|
| ZmeIF5A-1 Reverse | CGATCCCTGCACTTGTGTTT |
| ZmeIF5A-1 Forward | GCTATGGGGGAGGAGCAGAT |
| ZmeIF5A-2 Reverse | AGGGTTCCGTCTCCAAAGCCAAGG |
| ZmeIF5A-2 Forward | TCAGATGGCAACACTAAGGATGATCTT |
| ZmeIF5A-3 Forward | TGGAAAGGACCTCCTCCTGA |
| ZmeIF5A-3 Reverse | ACACCAACCACCTCGACAAA |
| RRB1 Forward | GCTGTTTCTGGTTATGTCTGTCCT |
| RRB1 Reverse | CTTTTGAGTACTTCTGTGCCTGAC |

Supplemental Figure S5.1: Sequences of primers used for cloning and RT-PCR.]

# CHAPTER SIX

## Conclusion

The pleiotropic effects of maize *opaque 2* mutation were investigated by both transcriptomic and proteomic analyses, which led to the identification of several lysine-rich proteins, such as GAPDH and SDH1. The elevated protein accumulation levels of these proteins could explain, in part, the high lysine content in *o2* endosperm. In addition, the data revealed changes in gene expression that could explain the increased susceptibility to insect and fungal attack as well as the soft and starchy endosperm texture of *o2* kernels. Several genes involved in plant defense, such as BGAF, RIP and CI-1B, were significantly down regulated in *o2*. The alteration of starch biosynthetic gene expression combined with the alteration of amylopectin branching patterns in *o2* starch could lead to the soft, starchy endosperm. This study identified specific candidate genes that can be further investigated to improve the nutritional quality and agronomic performance of high lysine maize lines.

Maize GCN2 kinase was cloned and 2 *Mu*-tagged *gcn2* alleles were used to characterize the role of GCN2 in response to amino acid starvation in maize endosperm and seedlings. GCN2 was shown to be essential when maize seedlings were treated with herbicides that inhibited amino acid biosynthesis, as *gcn2* mutant seedlings were more sensitive to the long term exposure. Phosphorylation of eIF2α by GCN2 regulated the translation of the maize transcription factor O2, indicating that O2 could be a GCN4 ortholog in maize. Although whether the O2 target transcript levels (especially those

involved in amino acid starvation) are increased during amino acid starvation still needs to be further investigated. A model of GCN2 kinase pathway in maize is proposed in Figure 6.1.

Surprisingly, maize *gcn2* mutant seedlings were found to be more tolerant to drought stress, contrary to the phenotype during amino acid starvation. *gcn2* leaves were found to have a significantly higher ABA abundance compared to wild type. When subjected to drought stress, the ABA levels in both genotypes appeared to be similar. A transcriptome study was performed to identify candidate genes that could explain the elevated ABA abundance as well as the increased drought tolerance in *gcn2*. Several ABA/drought inducible regulatory proteins were identified to be up regulated in *gcn2* leaves and might contribute to the increased drought tolerance. However, it is necessary to perform further analysis to identify genes involved in ABA biosynthesis and/or signaling that are altered in *gcn2*.

Finally, to better understand the synthesis and retention of γ-zein during the formation of PB in maize endosperm, γ-zein mRNA associated proteins were investigated and eIF5A was identified. eIF5A was found to be expressed in highly expressed in tissues of high levels of proliferation activity. Furthermore, eIF5A was found to be associated with an actin-rich cytoskeleton fraction by a pull-down analysis. Direct protein-protein interaction analyses confirmed that eIF5A was associated with F-actin, and this association is hypusine-dependent. In addition, eIF5A was also shown to be associated with EF1A and eIF2α. Together our data suggested that eIF5A may participate in promoting the association of γ-zein mRNA with the actin-rich cytoskeletal matrix that surrounds the endosperm PB ER.

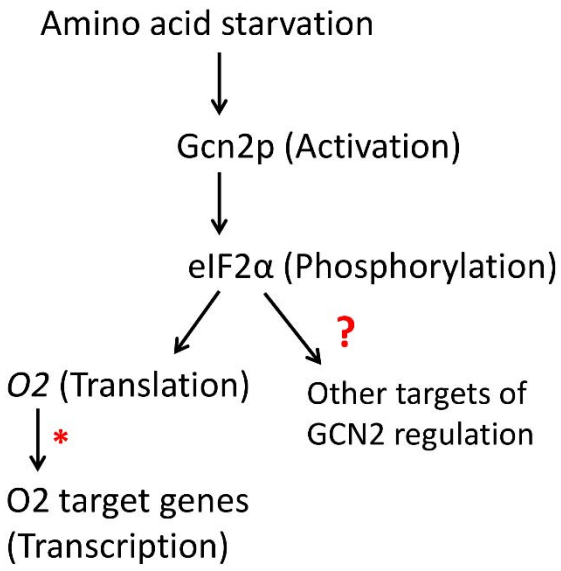


Figure 6.1: Proposed model for the maize general amino acid control pathway.

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