ABSTRACT

Role of GCN2 in Maize Cold Response

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Wild type maize is deficient in several key amino acids necessary for proper human nutrition. The maize *opaque2* mutation gives the grain a more complete protein content, but it makes the endosperm fragile. Lines known as Quality Protein Maize (QPM) have been created to combine enhanced protein content with stronger endosperm, but they are difficult to produce because they require the introgression of several *opaque2* modifier genes. A better understanding of the mechanisms that control the *opaque2* gene could help in the improvement of QPM. Translation of opaque2 is regulated by the protein kinase GCN2, which is activated by low amino acid levels. GCN2 activity has also been implicated in other environmental stresses, such as drought and extreme temperatures. This study aims to examine the relationship between GCN2 and the CBL/CIPK stress response pathway. Stress responses in plants often induce specific increases in cytosolic calcium levels, which are decoded by CBLs. CBLs signal forward to specific CIPKs, which enact stress response. This study analyzed expression of CBL4, CIPK16, CIPK17, and CIPK24 under conditions of cold stress in wild type and GCN2

mutant seedlings. Maize seedlings were grown in cold chambers, and leaf tissue samples were collected at several time points. GCN2 mutant plants thrived more effectively than wild type plants under cold conditions. Additionally, RNA was extracted from samples and used to synthesize cDNA. Expression of cold response genes was evaluated using qPCR. Expression of all cold response genes was increased in GCN2 mutant seedlings as compared to wild type seedlings grown at cold temperatures. This is indicative of a link between GCN2 activity and maize cold response.

APPROVED BY DIRECTOR OF HONORS THESIS

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ROLE OF GCN2 IN MAIZE COLD RESPONSE

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By

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CHAPTER ONE

Background

Opaque2 and Quality Protein Maize

Wild type maize (*Zea mays*) is deficient in several key amino acids—especially lysine and tryptophan— that are necessary for proper human nutrition (Gibbon and Larkins, 2005). As cereal grains make up a significant portion of the human diet worldwide, increasing the content of these amino acids could be widely beneficial. The basis for this incomplete amino acid content lies in the endosperm of maize kernels. Over 70% of the proteins that compose the endosperm are zein proteins— prolamin storage proteins that are poor in lysine (Gibbon and Larkins, 2005). A mutation known as *opaque2* has been identified in maize that increases lysine content by decreasing production of zein proteins and increasing expression of lysine-rich substitutes (Mertz and Nelson, 1964; Jia et al. 2013). This gives maize a more complete protein content, but due to the altered storage protein levels, it makes the kernel fragile and susceptible to damage by natural conditions. (Vasal et al., 1980).

Lines of *opaque2,* known as Quality Protein Maize (QPM), have been successfully created to combine more complete protein content with a stronger endosperm (Vasal et al., 1980). Due to the number of modifier genes that influence this line, however, development of QPM is a very time consuming process. First, *opaque2* mutants must be crossed with maize plants containing a wide array of genetic backgrounds. Resulting progeny that contain high lysine content, as well as vitreous

endosperm, are then selected and interbred (Vasal et al., 1980). This process is repeated for several cycles until the kernels possess high levels of favorable modifier genes. While these kernels now contain high levels of lysine and a strong, vitreous endosperm, they may still lack several agronomic factors necessary for a strong food crop. Thus, these modified *opaque2* mutants must then be crossed with elite inbred lines with strong agronomic characteristics (Vasal et al., 1980). Kernels are selected that still maintain vitreous and lysine-rich endosperm, but also have improved agronomic performance (Vasal et al., 1980). These selected kernels must then be backcrossed with the elite lines to further improve their yield. This selection continues for several cycles to produce lines of high-yield, modified *opaque2* maize with vitreous endosperm. When these lines are crossed, QPM is produced.

This process can span several growth seasons and is thus quite difficult to produce. While the development of QPM was a revolutionary advancement—so much so that its creators won the 2000 World Food Prize for their achievement—much more remains to be understood about the molecular basis for QPM. Examining the cellular mechanisms regulating the *Opaque2* gene can shed some light on how these favorable characteristics arise. A more complete understanding of the ways in which amino acid control mechanisms lead to altered protein composition and quality may enable scientists to harness these mechanisms to improve agronomic performance. This, in turn, could lead to more rapid creation of new QPM lines.

Translational regulation of opaque2

Opaque2 is a homolog of a gene in yeast that codes for the transcription factor general control nonderepressible 4 (GCN4). In yeast, GCN4 regulates expression of several amino acid biosynthesis enzymes in response to amino acid deprivation (Hinnebusch, 2005), which is one role of *Opaque2* in maize endosperm (Gibbon and Larkins, 2005). GCN4 is a master regulator of the general amino acid control system (GAAC) in yeast. Under conditions of amino acid deprivation, GCN4 has been found to upregulate several amino acid synthesis enzymes, various tRNA synthetases, purine biosynthetic enzymes, and other pathway activators (Hinnebusch, 2005). Almost one tenth of the yeast genome can be induced by GCN4 during amino acid starvation, some genes of which are directly related to amino acid control, but many that are not (Hinnebusch, 2005). Notably, stress conditions other than amino acid deprivation have also been found to induce the GAAC system (Hinnebusch, 2005). For this reason, the function and regulation of GCN4 are of particular interest.

Under normal conditions, translation of GCN4 is repressed by the presence of upstream open reading frames (uORFs) that bind to the small ribosomal subunit and initiate translation at a site upstream of the start of the GCN4 coding sequence (Hinnebusch, 2005). Four uORFs exist upstream of GCN4, of which uORF1 and uORF4 are necessary and sufficient for regulation of GCN4 translation (Hinnebusch, 2005). Generally, a ribosome will bind to and translate uORF1, then the translation machinery will dissociate and reform at the start codon of uORF4 (Figure 1A). After translating uORF4, however, the translation machinery cannot reform in time to bind the start codon of GCN4. Thus, GCN4 is not translated. Conditions of amino acid starvation can

stimulate translation of GCN4 by inducing phosphorylation of eIF2α, a member of the ternary complex (TC) required to bind the mRNA to the small ribosomal subunit and to recognize AUG start codons. Phosphorylation of eIF2α inhibits the formation of the TC. Due to this reduced TC creation, the ribosome skips translation of uORF4 and binds to the start codon of the GCN4 main ORF instead (Figure 1B) (Hinnebusch, 2005). Thus, GCN4 is translated and is then able to upregulate expression of several amino acid synthesis genes.

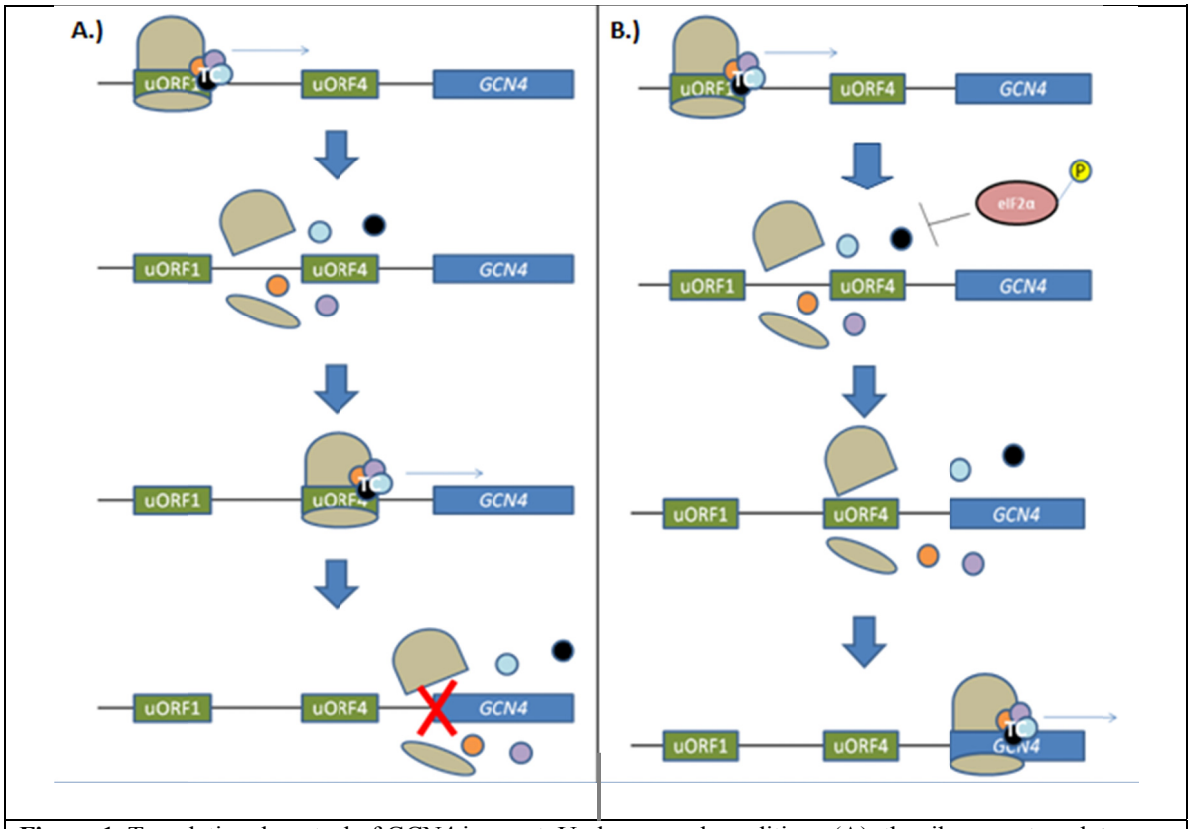


Figure 1. Translational control of GCN4 in yeast. Under normal conditions (A), the ribosome translates uORF1 and uORF4, but the translation machinery cannot reform in time to translate GCN4. Amino acid starvation (B), however, causes a delay in ternary complex formation. This causes the translation machinery to skip translation of uORF4 and reform instead around the start codon of GCN4.

In yeast, eIF2α is phosphorylated by the protein kinase GCN2 (Figure 2). GCN2 senses amino acid starvation within the cell by binding to uncharged tRNA molecules (Hinnebusch 2005) (Figure 3). The binding of uncharged tRNA activates the kinase activity of GCN2, allowing it to phosphorylate eIF2α. As GCN2 is a major regulator of GCN4 expression, it is of particular interest in the control of amino acid synthesis. Homologs of yeast GCN2 have been identified in several plant species, such as *Arabidopsis*, wheat, rice, and maize (Lageix et al., 2008; Byrne et al., 2012). In *Arabidopsis,* it has also been shown to phosphorylate eIF2α (Zhang 2008).

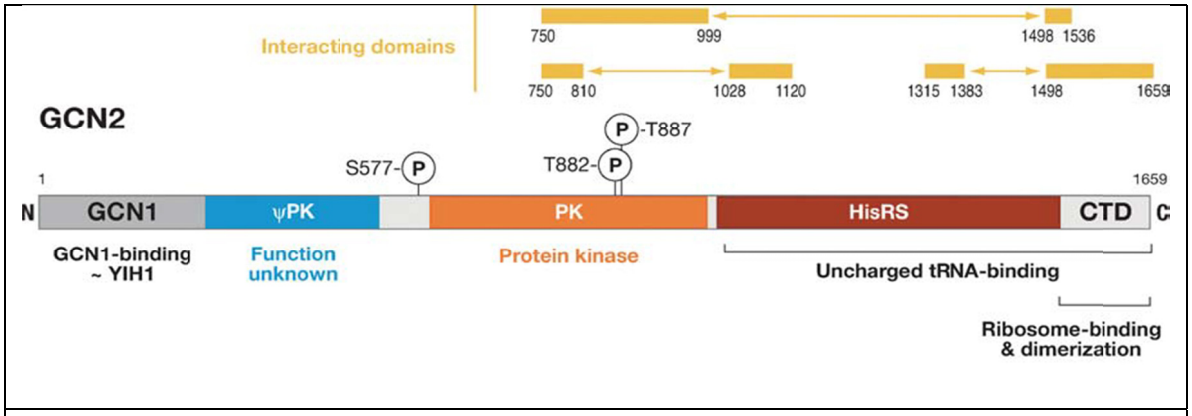


Figure 2. Figure adapted from Hinnebusch 2005 depicting a map of GCN2 functional domains. GCN2 contains four major domains: an N-terminal domain that binds to the positive regulatory factor GCN1, a pseudokinase domain with unknown function, the protein kinase domain responsible for phosphorylation of eIF2α, and the HisRS domain that binds to uncharged tRNAs.

GCN2 and environmental stress

The effects of environmental stresses on GCN2 expression and activity have been examined in plants. In wild type maize, GCN2 has been shown to be constitutively expressed in all tissues at all stages of development (Sekhon et al. 2011; Figure 4), suggesting that it must perform roles other than regulation of *Opaque2,* which is only

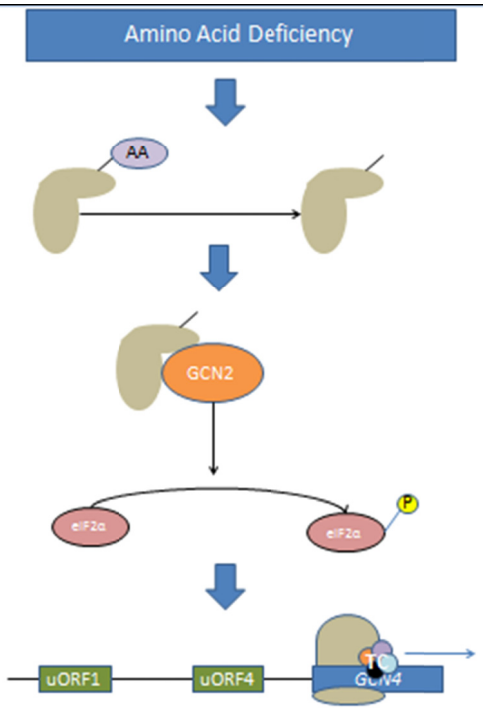


Figure 3. Model of GCN2 in the regulation of GCN4. Under conditions of amino acid starvation, the concentration of uncharged tRNA molecules increases. GCN2 binds to these uncharged tRNAs, activating its kinase activity. GCN2 then phosphorylates eIF2α, which upregulates expression of GCN4.

expressed in endosperm. Studies have shown that in *Arabidopsis*, mutants lacking the protein GCN2 were less able to overcome amino acid deficiencies than their wild type cohorts (Lageix et al., 2008). In addition, purine deprivation, exposure to UV radiation, tissue injury, and cold shock were met with a marked increase in *Arabidopsis* GCN2 activity (Lageix et al., 2008). In maize, *opaque2* mutants demonstrate an upregulation in several stress response genes. (Hunter et al., 2002; Jia et al., 2013). Additionally, our lab has shown that *opaque2* mutants show an increased ability to recover from cold shock. (Jia and Gibbon, unpublished). While these data clearly suggest a relationship between GCN2 and plant stress response, little has been studied about the biochemical basis for this correlation—especially in maize. This study aims to examine the link between GCN2 and a pathway that responds to several environmental stress responses in plants.

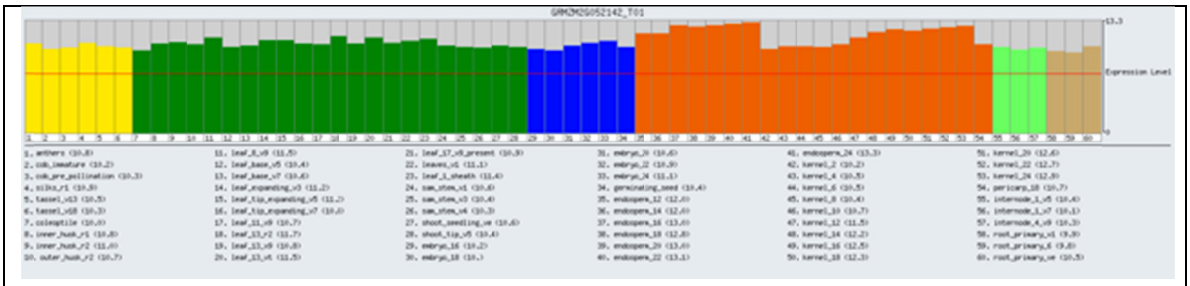


Figure 4. Image from Sekhon et al. 2011 showing the expression profile for maize GCN2. GCN2 is expressed in maize at consistent levels across all tissues and all stages of development.

The CBL/CIPK Pathway and Maize Stress Response

One key way that plants have been shown to respond to various environmental stresses is by regulation of cytosolic calcium (Ca^{2+}) levels. Many stresses— such as drought, heat, and cold shock—all use calcium ions as a second messenger to signal forward to individual stress response pathways. Each stress, however, produces a unique calcium response (Luan 2009; Chen et al. 2010). Molecules that detect these different responses are required to decode these unique signals.

One of the major classes of calcium sensors consists of calcineurin B-like proteins (CBLs), which bind to calcium with high affinity. (Luan 2009) Approximately 10 CBLs have been identified in rice and Arabidopsis, though very little has been studied about them in maize (Chen et al. 2010). These few CBLs can respond to a multitude of different stress signals, however, due to the immense number of downstream effectors present in the cascade. Different calcium signals induce different structural changes in CBL molecules, which allows for the activation of more specific downstream targets (Luan 2009; Figure 5).

These downstream targets form a class of molecules known as CBL interacting protein kinases (CIPKs). Consistent with the idea of increasing specificity, 43 distinct

CIPKs have been identified in maize (Chen et al. 2010). Expression of these CIPKs have been studied in response to many environmental stresses. Some CIPKs show increased or decreased expression in response to a single stress, while others respond to many stresses. Thus far, no studies have been performed to examine the relationship between GCN2 and CIPKs in plants. This study aims to examine whether a link exists between these pathways by investigating the response of GCN2 mutant seedlings to cold shock. We will examine gene expression levels of CBL4, CIPK16, CIPK17, and CIPK24 to determine which factors—if any—are affected by GCN2. We will also examine GCN2 expression and activity in maize grown under cold conditions by investigating levels of phosphorylated eIF2α in these plants.

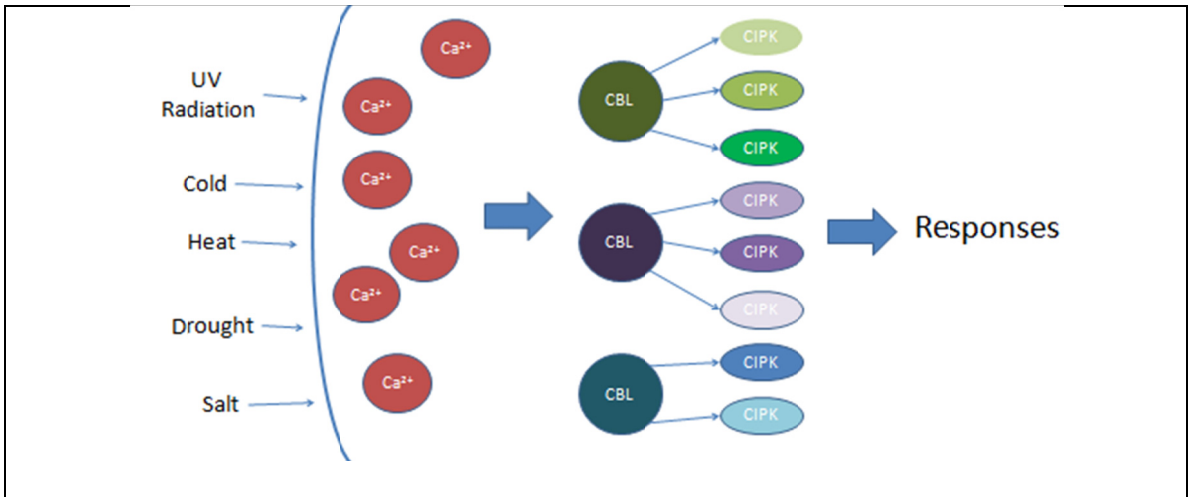


Figure 5. The CBL/CIPK pathway. Different environmental stresses promote various changes in cytosolic calcium levels. CBLs decode these signals and signal forward to a variety of CIPKs, which enact the appropriate response.

CHAPTER TWO

Materials and Methods

Seedling growth and sample preparation

Seventy-two wild type kernels and seventy-two *gcn2* mutant kernels were germinated in petri dishes for three days. Seeds that germinated successfully were then planted in soil in a 72-cell tray (1.5in x 1.5in x 2.25in cells; Growers Supply, Dryersville, IA). The seedlings were grown at room temperature for six additional days on a 16-hour light/8-hour dark cycle. After this time, the experimental group was transferred to a cold treatment chamber maintained at approximately 6°C, while the control group remained at room temperature. Both groups were maintained on the same light/dark cycle and watered regularly.

The first true leaf of each seedling was extracted and snap frozen in liquid nitrogen. Tissue samples were collected from control and experimental groups at 0, 1, 3, 6, 9, 12, and 24 hours, as well as 3 and 7 days after transfer to the cold chamber. Samples were stored at -80°C.

Cold tolerance and recovery testing

Twelve wild type and twelve *gcn2* seedlings were germinated and planted as described above. Five days after planting, the experimental group was transferred to the cold treatment chamber (6°C) for 5 days. Photographs of each group were taken at two and five days after the start of treatment using a Nikon D5000 digital camera. Five days after

the start of treatment, the cold-treated seedlings were removed from the treatment chamber and returned to room temperature. They were photographed at two and five days to evaluate differences in their ability to thrive after treatment.

RNA isolation

Tissue samples were snap frozen in liquid nitrogen then ground using small pestles. Approximately 50mg of sample were weighed out and added to 0.5mL TRIzol Reagent (Life Technologies, Grand Island, NY). RNA was isolated from the TRIzol solution using the Direct-zol™ RNA MiniPrep kit (Zymo Research, Irvine, CA) as instructed by the kit protocol. The concentration of the resulting RNA was evaluated using the NanoDrop ND-1000 UV/Vis Spectrophotometer (NanoDrop Technologies, Wilmington, DE) according to manufacturer's instructions.

Reverse transcription and qPCR

cDNA was synthesized from ~0.5µg isolated RNA using qScript™ cDNA SuperMix (Quanta Biosciences, Gaithersburg, MD). The resulting cDNA was diluted 10-fold with DI water. Primers for qPCR were designed using Primer3 Plus software. The primers used are listed below in Table 1.

Each qPCR reaction mixture was composed of 10µl SYBR® Green FastMix® (Bio-Rad, Hercules, CA), 2.5µl diluted cDNA, and 1µM concentrations of forward and reverse primer. The total reaction volume was then brought up to 20µl using DI water. The reaction was performed using the Corbett Rotor-Gene 6000 (Qiagen, Valencia, CA). The program followed is described in Table 2. A melting point curve was obtained by heating from 55°C to 95°C at a rate of 1°C per second to confirm amplicon purity.

Table 1. Primers for qPCR

Primer	Sequence
RRB1	F: 5'GCTGTTTCTGGTTATGTCTGTCCT3' R: 5'CTTTTGAGTACTTCTGTGCCTGAC3'
CBL4	F: 5'TCGTGCGGTCGCTCAGTGTGTT3' R: 5'ATGCACTCTGCTGGCCGTTGCT3'
CIPK16	F: 5'GTGCTCTACGTCCTGCTCTG3' R: 5'CGTTTCTTGGGCGTCATCG3'
CIPK17	F: 5'AACATCTCGGGAACGATGGGTT3' R: 5'GGAGGAAGGACAGGGACGTAGTG3'
CIPK24	F: 5'GAATGCCTTTGAGATGATTACGC3' R: 5'CTTCAACCATAACTGAGAGATGA3'

Table 2. Thermocycler Program for qPCR

Number of Cycles	Time	Temperature
1	2 min	50°C
1	10 min	95°C
50	15s	95°C
	1min	60°C

To confirm the size of the amplified fragments, qPCR product was run on a 1% (w/v) agarose gel (100V for ~30min) and imaged using the Ultra-LUM Gel Imager and UltraQuant 6.0 Software (UltraLum, Claremont, CA).

SDS-PAGE and Western blotting

Total protein extracts were collected using a 10% trichloroacetic acid (TCA) solution (10% TCA with 0.3% DTT in acetone) from both treated and untreated wild type and *gcn2* seedlings. Twenty-five µg of total protein from each sample were loaded into a

12.5% SDS-PAGE gel and run in 1X SDS-PAGE running buffer (200V for ~1h). Protein was then transferred to a BioTrace™ PVDF membrane (Pall Corporation, Pensacola, FL) in 1X SDS-PAGE transfer buffer (60V for 1 hour). Transfer was confirmed by staining with Ponceau S and subsequent destaining with 1X TBST. The membrane was blocked with 3% (w/v) bovine serum albumin in TBST at room temperature for 1 hour with shaking.

Membranes were then incubated with primary antibody at room temperature for 1-2 hours. After rinsing with TBST, blots were incubated with secondary antibody for 30 minutes at room temperature. All dilution information for each antibody is listed below in Table 3. Blots were washed again with TBST then incubated with 1mL SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL) for 2 minutes at room temperature. The blot was imaged using the ImageQuant LAS 4000 imager (GE, Fairfield, CT).

Table 3. Antibody Information

Antibody	Dilution Factor	Animal
GCN2	1:1000	Rabbit
eIF2α	1:5000	Rabbit
p-eIF2α	1:2000	Rabbit
Actin	1:2000	Mouse

CHAPTER THREE

Results

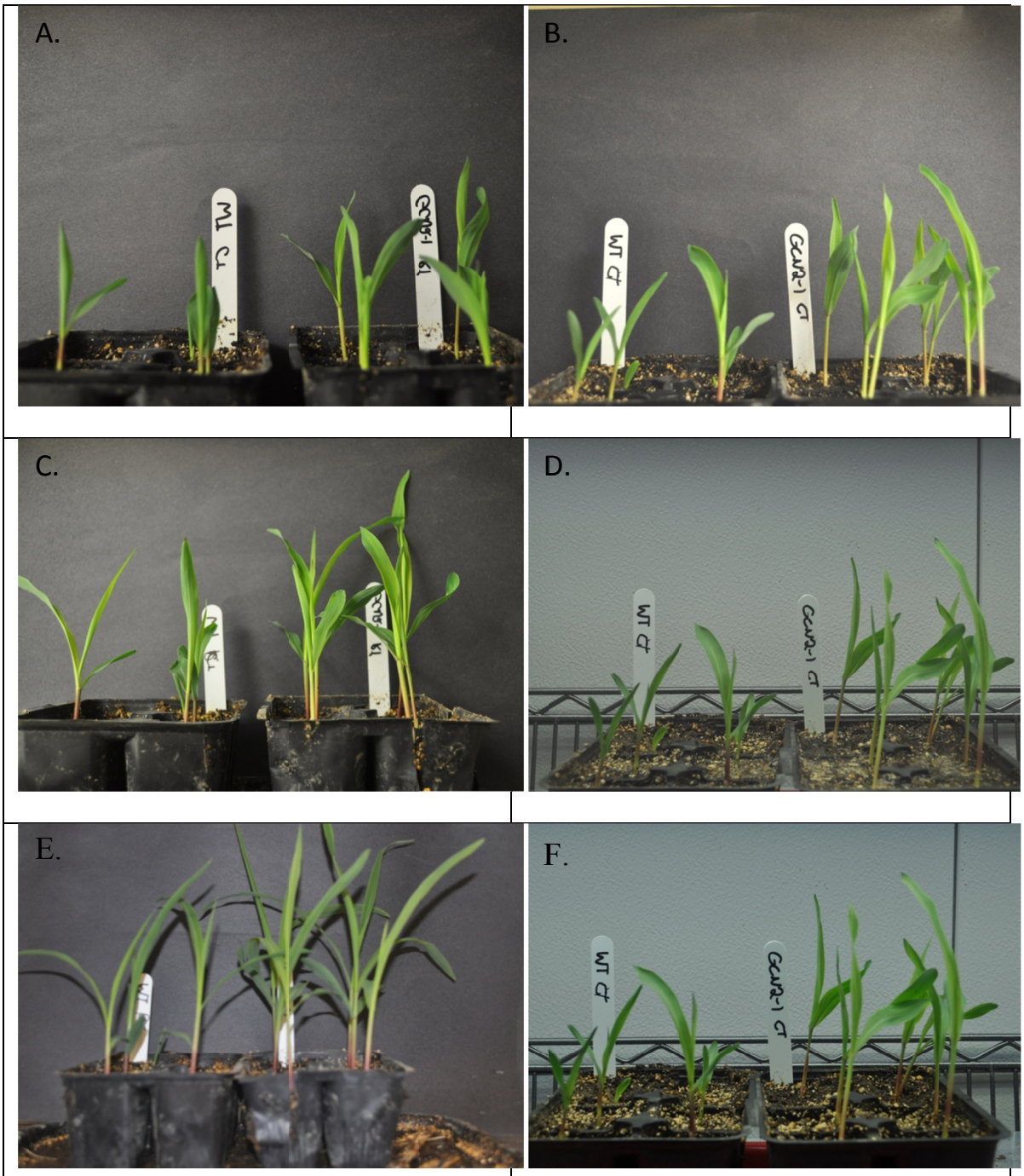
GCN2 mutant seedlings respond more effectively to cold shock than wild type seedlings

Responses of wild type and GCN2 mutant seedlings to cold shock were examined qualitatively. At 48 hours after introduction to cold conditions, both wild type and mutant seedlings showed a decreased height of several centimeters as compared to their untreated counterparts. Differences were also visible between the height of wild type and mutant seedlings. Mutant seedlings exposed to cold shock were significantly taller than wild type seedlings exposed to the same treatment. Though this same difference was also observed at room temperature, it was far more pronounced in the treated seedlings.

At five days after the beginning of treatment, differences in height between the wild type and mutant seedlings were even more pronounced in the cold-treated samples. Images of the seedlings during treatment are shown in Figure 6.

GCN2 mutant seedlings recover more effectively from cold shock than wild type seedlings

The cold-treated seedlings were later returned to room temperature to evaluate differences in their ability to thrive after treatment. Two days after removal from the cold chamber, the wild type plants began to show signs of withering, namely tissue death at the leaf tips. The mutant seedlings, however, showed no significant damage. By five days after the return to room temperature, the withering of wild type plants was more



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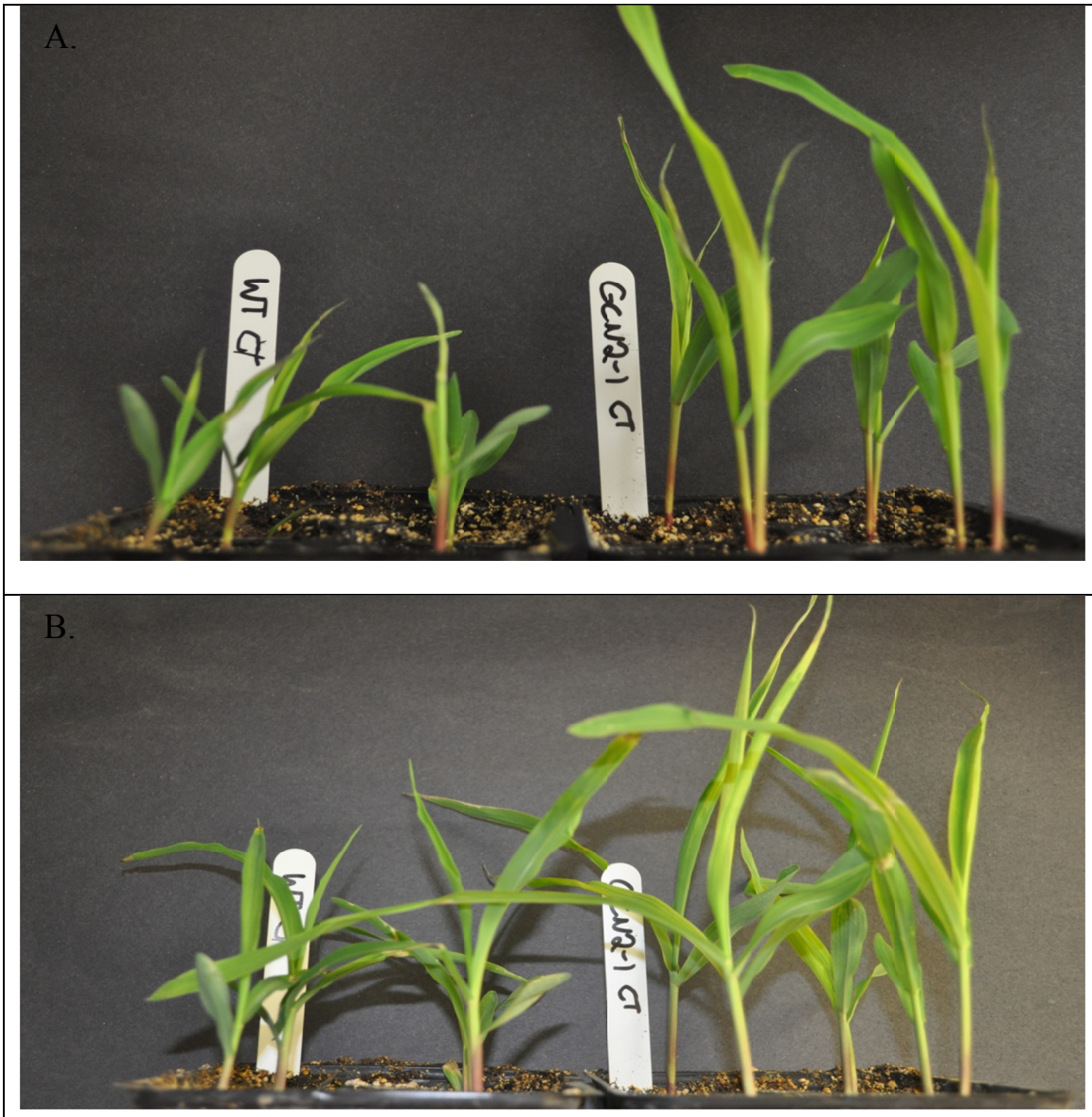

Figure 7. Cold-treated seedlings two days (A) and seven days (B) after removal from cold-treatment chambers. At two days after return to room temperature, the wild type plants showed signs of withering at the leaf tips, while the mutant seedlings continued to grow. At five days after treatment, the wild type seedlings showed growth as well, but more severe signs of tissue death were evident on the leaves. The mutant plants continued to thrive.

pronounced. Mutant seedlings appeared, at this point, nearly as tall and healthy as their untreated counterparts. Images of these plants are shown in Figure 7.

Expression of CIPK genes is upregulated in GCN2 mutant seedlings as compared to wild type seedlings.

Expression of several CIPK genes was evaluated using qPCR. Relative expression of each gene was standardized against the housekeeping gene RRB1. Expression of genes within each experimental group was measured across a time course from 0 to 6 hours after the start of cold treatment (Figure 7). In all groups, expression began low at the 0-hour time point. A sharp increase in expression of all examined genes occurred at the 1-hour time point in all experimental groups. Expression then decreased again and leveled out at the 3 and 6-hour time points. This trend is shown below for expression of CBL4. All genes, however, showed a similar trend (data not shown).

For every time point except the 1-hour point, expression levels did not vary between the experimental groups. The sharp increase in expression noted during the one hour time point, however, was most dramatically observed in the mutant cold-treated plants. For CBL4, CIPK16, and CIPK17, expression in the mutant cold-treated seedlings exceeded that of the wild type cold-treated seedlings at least 6-fold (Figure 8). In CIPK24, expression was similar between mutant and wild type plants. Though the differences did not reach statistical significance for any gene, a clear trend is present.

This drastic difference in CBL/CIPK expression between wild type and mutant seedlings provides a possible explanation for the enhanced ability of GCN2 mutants to recover from cold shock. These data suggest a possible regulatory role of GCN2 in maize cold response.

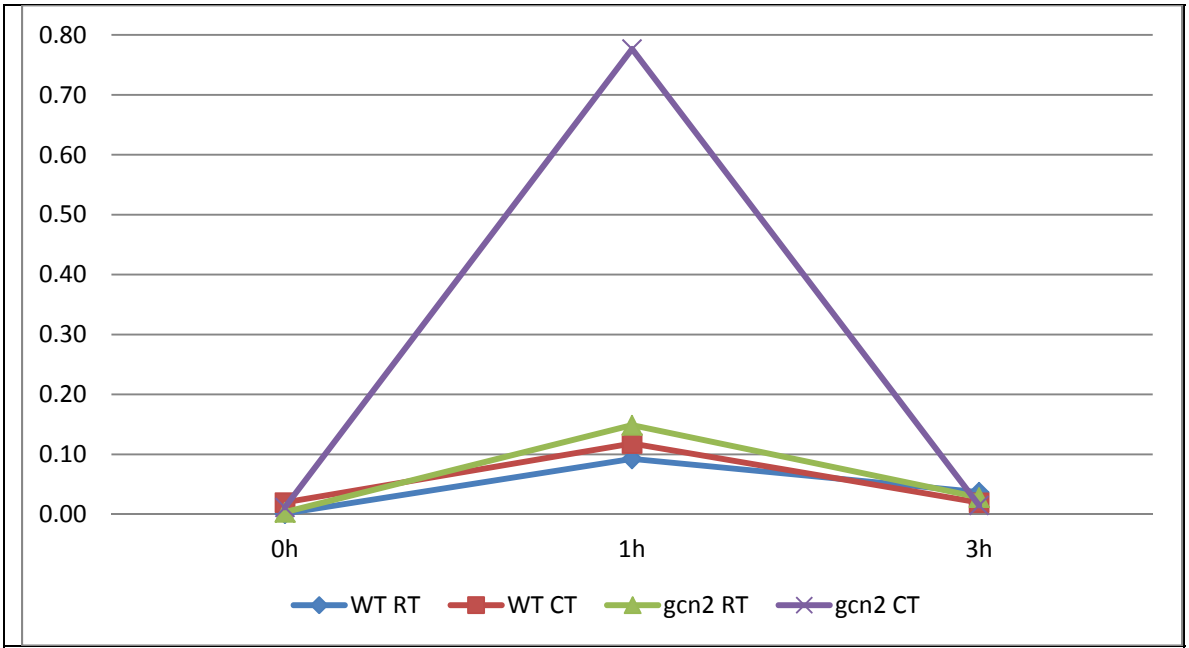

Figure 7. Relative expression of CBL4 among all types of seedlings. Expression increased dramatically in all groups at the 1 hour time point, then decreased again and leveled off. This increase was most pronounced in the cold-treated mutant seedlings. Data was similar for all examined elements of the CIPK pathway (data not shown).

Expression of GCN2 and phosphorylated eIF2α

Analysis of expression and activity of GCN2 in response to cold conditions was attempted by means of Western blot. Total protein was isolated from wild type leaf samples collected during the experiment and run on an SDS-PAGE gel. Antibodies against GCN2, eIF2α, phosphorylated eIF2α (p-eIF2α), and actin (as a loading control) were used to probe for the expression of the protein. Results were inconclusive due to time constraints. Expression of neither GCN2 nor eIF2α is expected to change in response to cold treatment. In untreated samples, no detectable amount of p-eIF2α is expected, as eIF2α is activated only under stress conditions. Levels of p-eIF2α, however, are expected to increase in cold-treated seedlings as an indicator of GCN2 activity.

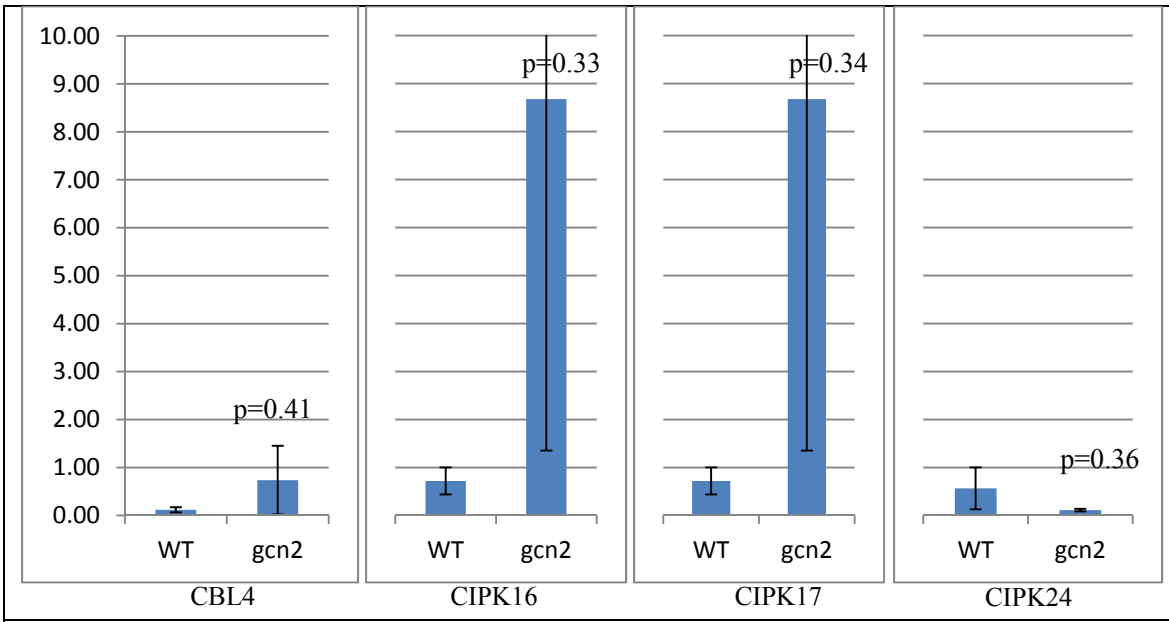

Figure 8. Relative expression of elements of the CIPK pathway one hour after exposure to cold. In three of the four elements, a dramatic spike in expression is seen in mutant seedlings as compared to wild type seedlings. Listed p-values were determined using Student's T-test. No comparisons reached statistical significance.

CHAPTER FOUR

Discussion

The purpose of this study was to examine a possible link between GCN2 activity and the cold response pathway in maize. This was accomplished this by examining differences in the ability of wild type and GCN2 mutant seedlings to respond to cold shock—at both the molecular and organism level. Key differences in the responses of the two groups were observed in both cases.

At the organism level, we observed the physical reaction of both groups of seedlings to cold temperatures. The mutant seedlings thrived considerably better under conditions of cold shock than their wild type counterparts. At the end of the five-day cold exposure period, mutant seedlings were noticeably taller and more developed. This indicated a clear link between GCN2 and the maize cold response pathway. Additionally, it suggested that seedlings respond more readily to cold shock in the absence of functional GCN2 protein.

This correlation was also observed at the molecular level. CBLs and CIPKs are known indicators of many environmental stresses, such as cold response (Chen et al. 2010). The CIPKs examined in this study have all been shown to be upregulated in response to cold treatment in wild type plants (Luan 2009). This study examined how a mutation in GCN2 altered this expression response.

At the 1-hour time point, expression of all tested CIPKs was increased in all experimental groups, regardless of genotype or treatment. The basis for this increase in

untreated plants is unknown and requires further investigation. It is clear, however, that for all genes except CIPK24, the most drastic increase in expression was observed in the cold-treated mutant plants. The difference in expression between cold-treated wild type and mutant plants was not significant ($p>0.3$ in all cases) due to the large amount of deviation present in the data. C_t values for RRB1 were fairly consistent at the 1-hour time point. However, C_t values for all CIPKs varied widely. Loading inconsistencies in the qPCR run could have contributed to the large amount of error. Additionally, the small sample size used in the experiment likely enhanced minor differences between individual plants. Time allowing, this qPCR analysis should be repeated. This trend should also be further examined in a larger cohort of seedlings.

This molecular data supports the observation that GCN2 mutant seedlings have an increased ability to recover from cold shock. By placing these observations together, we can see that a mutation in GCN2 confers an increased response to cold stress. It upregulates expression of certain cold response genes, which in turn improves a seedling's ability to respond to cold shock.

The upregulation of CIPK levels in GCN2 mutants suggests a regulatory role of GCN2 in CIPK expression. In the absence of GCN2, CIPKs are constitutively expressed (Jia and Gibbon, unpublished). Mutant seedlings therefore demonstrated an increased ability to respond to cold. As stated earlier, GCN2 activity in Arabidopsis has been observed to increase drastically in response to several environmental stresses—including cold shock (Lageix et al. 2008). These data support the same trend in maize cold response.

These data make it clear that GCN2 activity must also regulate expression of genes other than *opaque2*. This observation is reasonable, considering the fact that *opaque2* is only expressed in endosperm, while GCN2 is expressed globally (Gibbon and Larkins 2005; Sekhon et al. 2011). Other mechanisms must be regulated by GCN2 at other stages of development. As stated earlier, maize *opaque2* mutations have been linked to overexpression of many genes involved in stress response (Hunter et al. 2002; Jia et al. 2013). Possibly, the regulatory effects of this pathway on stress response genes act as a conservation mechanism. This idea is supported by the observation that activation of GCN2 decreases universal protein synthesis in Arabidopsis (Lageix et al. 2008). The GAAC pathway is generally activated by a deprivation of amino acids. It stands to reason that, if the cell is deprived, it would conserve amino acids by down regulating expression of proteins that are less necessary.

A better understanding of the mechanisms that relate the GAAC pathway to the cold response pathway and other environmental stress response pathways would be beneficial in gaining a more complete understanding of the regulation of *opaque2*. This study established that expression of GCN2 is not altered by cold shock. The changes in the GAAC pathway as a result of cold shock are due to an increase in GCN2 activity alone. It is known that GCN2 is activated by the presence of uncharged tRNAs, which denote a deficiency in amino acid concentrations (Hinnebusch 2005). However, GCN2 activity is also known to dramatically increase in response to several environmental stresses (Lageix et al. 2008). This suggests that factors other than amino acid deficiency must also activate the pathway.

In Arabidopsis, purine deprivation and UV irradiation lead to rapid increases (<3 hours) in GCN2 activity (Lageix et al. 2008). One hypothesis concerning the mechanism of activation in these cases is that these stresses could lead to decreased tRNA aminoacylation efficiency, causing a buildup of uncharged tRNAs. In yeast, GCN2 is also known to be constitutively activated by dephosphorylation of the Ser-51 residue (Hinnebusch 2005). It is possible that cold shock could interfere with the autophosphorylation mechanisms responsible for phosphorylating Ser-51. Alternatively, cold exposure could activate a phosphatase that removes this phosphate. Rapamycin, an inhibitor of the mTOR pathway, has been shown to inhibit Ser-51 phosphorylation in yeast (Hinnebusch 2005). However, the relationship between mTOR and GCN2 does not seem to be affected by stress response in Arabidopsis (Lageix et al. 2008). The mechanisms by which stress responses regulate GCN2 activity must be further explored.

To better understand the ways in which the GAAC pathway impacts maize cold response specifically, the mechanism of interaction between the two must be characterized. This study has proven that a link exists between these pathways, but to gain a full understanding of this link, other elements of maize cold response must be examined. While CIPKs are implicated in several various environmental stresses, the effects on expression of genes specific to the maize cold response pathway must also be studied. By repeating these experiments on well-characterized members of the cold response pathway, it may be possible to determine where the GAAC pathway intersects the cold response pathway.

It would also be worthwhile to characterize how, on the molecular level, the GAAC pathway interacts with other common environmental stress response pathways,

such as drought, salt, and heat. As seen in Arabidopsis, GCN2 impacts many stress response pathways other than cold response (Lageix et al. 2008). The observation that GCN2 mutant seedlings recover more effectively from cold shock than wild type seedlings also suggests a link between GCN2 and the heat response pathway. To further evaluate the basis for this response, the heat response pathway must also be explored in depth using a similar experimental design. Several different environmental stress pathways can be triggered by the same mechanisms, as seen in the way CIPKs respond to changing calcium levels. By examining GCN2 interaction with various stress response pathways, some commonalities may arise that could provide more information about the GAAC pathway.

By characterizing the many ways the GAAC pathway responds to environmental stresses, we can gain a better understanding of how it is regulated, and how it regulates other elements of the cell. A deeper understanding of this pathway is crucial to the efforts to improve agronomic performance of maize. Understanding the ways in which maize plants respond to various environmental conditions could help improve the way maize is grown in certain environments. By delving into the regulatory mechanisms of these stress response pathways, the ways in which maize will respond to new environments and changing climates could be predicted. It may be possible to identify mutations that would allow for the growth of maize in environments that are extremely cold, warm, dry, or otherwise unfavorable. An understanding of the relationship between the GAAC pathway and these stress responses could potentially better the agronomic performance of QPM with a less arduous breeding process. Though much work on this complicated process

remains to be done, a more complete characterization of the interaction between GCN2 and environmental stress could yield many important agronomic benefits.

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