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Potential Roles of WRKY Transcription Factors in Resistance to *Aspergillus flavus* Colonization of Immature Maize Kernels

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POTENTIAL ROLES OF WRKY TRANSCRIPTION FACTORS IN RESISTANCE TO *ASPERGILLUS FLAVUS* COLONIZATION OF IMMATURE MAIZE KERNELS

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To my father and mother, Larry Fountain and Penny Rodgers.

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LIST OF ABBREVIATIONS

ABA	Abscisic Acid
ACC	1-aminocyclopropane-1-carboxylic acid
AF	Aflatoxin
At	*Arabidopsis thaliana*
ASC	ACC Synthase
BLAST	Basic Local Alignment Search Tool
cDNA	Complementary DNA
Ct	Threshold Cycle
DAI	Days After Inoculation
DAP	Days After Pollination
ERF1	Ethylene Responsive Factor 1
Hv	*Hordeum vulgare*
IAA	Indole-3 Acetic Acid
JA	Jasmonic Acid
kDa	Kilodalton
MAPK	Mitogen Activated Protein Kinase
MAP2K	MAP Kinase Kinase
MAP3K	MAP Kinase Kinase Kinase
MEKK1	MAP Kinase Kinase Kinase 1
MKS1	MAP Kinase 4 Substrate 1
MPK4	MAP Kinase 4
NCBI	National Center for Biotechnology Information

NPR1	Non-Expressor of Pathogenesis-Related Protein 1
Os	*Oryza sativa*
PAMP	Pathogen Associated Molecular Pattern
Pc	*Petroselinum crispum*
ppb	Parts Per Billion
PR	Pathogenesis-Related Protein
PTI	PAMP-Triggered Immunity
qPCR	Real Time/Quantitative Polymerase Chain Reaction
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
rRNA	Ribosomal RNA
SA	Salicylic Acid
SAR	Systemic Acquired Resistance
TF	Transcription Factor
Tm	Melting Temperature
Vv	*Vitis vinifera*
Zm	*Zea mays*

ABSTRACT

The infection and colonization of maize (*Zea mays* L.) by the necrotrophic fungal pathogen *Aspergillus flavus* results in contamination of kernel tissues with carcinogenic mycotoxins known as aflatoxins, resulting in severe economic losses as well as negative effects on human and animal health. Resistance to *A. flavus* is mediated by both inducible and constitutively expressed defense proteins; however the mechanism regulating the expression of these defenses is poorly understood. This study examined the potential roles of six maize WRKY transcription factors, *Zm*WRKY19, *Zm*WRKY21, *Zm*WRKY53, *Zm*WRKY53.1, *Zm*WRKY67, and *Zm*WRKY68, in regulating defense responses against *A. flavus*. The responses of these WRKY transcription factors to *A. flavus* inoculation were examined over a time course in the immature kernel tissues of two maize lines, B73 (susceptible) and TZAR101 (resistant) using real-time quantitative PCR. Three defense genes, Nonexpressor of Pathogenesis-Related Protein 1 (*ZmNPR1*), Pathogenesis-Related Protein 1 (*ZmPR-1*), and Ethylene Responsive Factor 1 (*ZmERF1*), were also examined in order to determine whether salicylic acid, jasmonic acid, or ethylene-mediated defense mechanisms were induced in response to *A. flavus* inoculation. The genes *ZmWRKY19*, *ZmWRKY53*, and *ZmWRKY67* were found to be induced by inoculation and constitutively expressed at higher levels in the resistant maize line. Both the putative *Zm*WRKY19 and *Zm*WRKY53 are homologs of *Arabidopsis* WRKY53 and WRKY33, respectively, with *Zm*WRKY53 also being homologous to rice and wheat WRKY53. These genes may function in promoting antioxidant enzymes to sequester reactive oxygen species (ROS) during pathogen infection or abiotic stress. *Zm*WRKY67 is homologous to *At*WRKY50 and may function in the suppression of jasmonic acid-regulated defenses in the resistant maize line. The expression of *ZmNPR1* was also induced by inoculation in the resistant variety without

concurrent induction of *ZmPR-1*, possibly due to the observed induction of *ZmERF1* and, therefore, ethylene-based defenses. These findings indicate that resistant maize lines may possess elevated oxidative stress tolerance potentially conferring resistance to programmed cell death as part of the hypersensitive response induced by ROS during necrotrophic pathogen infections. Future studies of these WRKY transcription factors are necessary to better understand their regulation and involvement in resistance to *A. flavus* infection and aflatoxin production.

1. INTRODUCTION

The infection of maize (*Zea mays* L.) by the fungal pathogen *Aspergillus flavus* results in the contamination of kernel tissues with carcinogenic mycotoxins produced during fungal secondary metabolism known as aflatoxins (Scully et al. 2009). The contamination of maize kernels with aflatoxin poses a significant threat to human and livestock health and results in substantial economic losses annually (Payne and Widstrom, 1992; Schmale and Munkvold, 2011; Shephard 2008). Maize resistance to *A. flavus* infection is mediated by various defense proteins including β-1,3-glucanases, chitinases, glyoxalase I (GLX-I), pathogenesis-related proteins 10 and 10.1 (*Zm*PR10 and *Zm*PR10.1), ribosome inactivating proteins, and zeamatin (Chen et al. 2004, 2006, 2010; Huynh et al. 1992; Guo et al. 1997; Lozovaya et al. 1998; Mauch et al. 1988; Walsh et al. 1991; Xie et al. 2010). In addition, maize varieties resistant to *A. flavus* infection have also been found to accumulate various antioxidant proteins including ascorbate peroxidase and superoxide dismutase (Pechanova et al. 2011), peroxiredoxin antioxidant (PER1) protein (Chen et al., 2007), and drought tolerance proteins such as late embryogenesis abundant proteins (LEA 3 and LEA 14) and osmo/salt-stress related proteins such as WSI18 (Chen et al. 2004). The presence of these potential abiotic stress resistance proteins corresponds to the observed correlation between abiotic stress tolerance and *A. flavus* resistance in maize (Kebede et al. 2012).

Although the identities of many defense proteins are currently known, the mechanism regulating their expression has yet to be fully understood. In plants, several signaling pathways mediated through phytohormones such as jasmonic acid (JA), salicylic acid (SA), and ethylene regulate the expression of various defense genes against biotic and abiotic stress (Glazebrook, 2005). Many components of these pathways are regulated by transcription factors of a type

known as WRKY transcription factors (Rushton et al. 2010). These transcription factors are characterized by their distinctive DNA binding domains which consist of approximately 60 residues containing an amino acid sequence of WRKY at the N-terminus of the domain, and a zinc finger structure at the C-terminus (Rushton et al. 1996, 2010). This binding domain binds promoter regions referred to as W-boxes with sequence of C/TTGACC/T (Euglem and Somssich, 2007). Based on key structural features and phylogenetic analyses, WRKY transcription factors can be compiled into seven unique groups with varying functions (Chen et al. 2012b; Rushton et al. 2010; Zhang and Wang, 2005).

Several WRKY transcription factors have been found to regulate the expression of defense in the model plant species *Arabidopsis thaliana*. Examples of this include *At*WRKY33, which is required for the induction of JA-promoted defense genes such as defensins (PDF1.1 and PDF1.2) and in ethylene biosynthesis (Birkenbihl and Somssich, 2011; Birkenbihl et al. 2012; Li et al. 2012), *At*WRKY11 and *At*WRKY17, which promote JA biosynthesis and suppress SA-induced basal defenses (Journot-Catalino et al. 2006), and *At*WRKY50 and *At*WRKY51, which suppress JA-based defenses (e.g. PDF1.2) and promote SA-based defense genes in the presence of reduced levels of oleic acid (Gao et al. 2011). WRKY transcription factors have also been found to be involved in plant responses to abiotic stresses such as heat stress and oxidative stress, such as *At*WRKY25, -26, -33, -39, and -53 (Li et al. 2010, 2011; Miao et al. 2004).

In order to better understand the mechanism of defense gene induction in maize in response to *A. flavus* infection, particularly the role of WRKY transcription factors, we examined the findings of a recent series of microarray analyses by Luo et al. (2011) of a resistant and a susceptible maize line derived from a cross between 1638 and GT-MAS:gk, Eyl25 and Eyl31, respectively (Menkir et al. 2006, 2008), to identify WRKY transcription factors differentially

regulated by between the two lines. We selected several candidate WRKY genes based on the above microarray study and examined their expression in the resistant and susceptible maize varieties TZAR101 and B73 over a time course of infection in Louisiana field conditions using real-time/quantitative polymerase chain reaction (qPCR). In addition, the expression levels of several indicator genes, NPR1 and PR-1, which regulate SA-based defenses (Spoel et al. 2007; Zhang et al. 1999), and ethylene responsive factor1 (ERF1), which regulates JA and ethylene-based defenses (Lorenzo et al. 2003), were also examined in order to determine which major phytohormone defense pathways were regulated by *A. flavus* infection.

2. REVIEW OF LITERATURE

2.1 *Aspergillus flavus* and Aflatoxins

Maize (*Zea mays* L.) is an important crop not only in Louisiana and the United States as a whole, but also for the world. Maize is a staple food crop for many developing countries, and also serves as a major component of livestock and animal feeds. It is also utilized in industrial and energy production applications. The infection and colonization of maize kernels by the opportunistic fungal pathogen *Aspergillus flavus* (Anamorph; *Petromyces flavus*, Teleomorph) (Horn et al. 2009) may result in the accumulation and subsequent contamination of maize kernel tissues with aflatoxins (Scully et al. 2009). Contamination of crops with aflatoxins is a serious agricultural problem resulting in a high degree of economic losses. Aflatoxins are carcinogenic mycotoxins known to cause numerous diseases in both humans and domesticated animals including aflatoxicosis, cirrhosis, hepatitis, liver cancer, and reproductive defects (Shephard 2008); as a result, interstate commerce of grains contaminated with aflatoxins higher than 20 ppb is prohibited (Payne and Widstrom, 1992). In the United States, aflatoxin contamination results in approximately $225 million in losses in maize annually (Schmale and Munkvold, 2011).

Aflatoxins are secondary polyketide-derived furanocoumarin metabolites produced mainly by *A. flavus* and *Aspergillus parasiticus* (Bennett and Klich, 2003; Chanda et al. 2009). There are six structural variations of aflatoxins including AFB_1, AFB_2, AFG_1, AFG_2, AFM_1, and AFM_2. Only aflatoxins AFB_1 and AFB_2 are produced naturally by *A. flavus*, and these B aflatoxins account for 90% of the observed aflatoxins in samples collected from naturally infected maize (Diener et al. 1987). Both $AFB_{1,2}$ and $AFG_{1,2}$ are produced through a common biosynthetic pathway with the diversion into either class occurring later in the process (Cleveland et al. 2004). Recent studies have determined that both early and late stages of

production of AFB_1 occur in vesicles in *A. parasiticus* within the fungal mycelia termed aflatoxisomes and that such structures may serve as the basis for the exocytosis and secretion of aflatoxins from *A. parasiticus* and, presumably, *A. flavus* (Chanda et al. 2009, 2010; Linz et al. 2011). Such secreted aflatoxins contaminate maize and other susceptible grain crops during the process of infection by *A. flavus* and subsequent colonization.

2.2 Life Cycle and Infection Strategy

Aspergillus flavus primary inoculum is typically produced from sclerotia which overwinter in the soil or debris following harvest in the previous season (Wicklow et al. 1984). Conidiophores produced by the proliferating sclerotial mycelia in favorable environmental conditions provide conidia as primary inocula. These conidia can be transmitted to maize by several means including wind, rain splash, or insects such as corn earworm (*Helicoverpa zea*), maize weevil (*Sitophilus zeamais*), or brown stink bug (*Euschistus servus*) (Diener et al. 1987; McMillian et al. 1980; Ni et al. 2011). Conidia introduced to maize silks by artificial inoculation techniques have been shown to germinate and grow rapidly down the silk from the ear tip to the base in as little as 4 to 13 days after inoculation (Marsh and Payne, 1984a).

Upon reaching the kernels after growing down silks, the mycelia begin the process of invading the kernels. Earlier studies seem to indicate that *A. flavus* can infect maize kernels through the stylar canal and cross the stylar abscission zone into the kernel (Jones, 1979). A study by Marsh and Payne (1984b) using scanning electron microscopy (SEM) noted localized conidiation around the silk tip region of kernels. However, SEM results did not show any signs of infection within the kernel below this region (Diener et al. 1987; Marsh and Payne, 1984b). A more recent study by Windham and Williams (2007) of various inoculation methods indicated that silk-based routes are not the primary methods of infection of maize kernels by *A. flavus*.

They determined that this was due to a lower degree of success in fungal colonization and aflatoxin accumulation using silk inoculation methods in comparison to pinbar and side injection inoculation techniques, which also damage the pericarp allowing for direct entry of fungal mycelia. These observations are supported by earlier findings which showed that although later stage, yellow-brown silk are the best medium allowing for *A. flavus* mycelial growth, senescence and detachment of these silks may reduce the probability of silk scar infection (Marsh and Payne, 1984a; Payne 1986).

Aspergillus flavus mycelia were also found to grow along the surface of the pericarp to the pedicel region, and are then able to infect the kernel through the adaxial zone of the rachilla at the glume insertion site of the kernel. This resulted in the invasion of the maize kernel intercellular spaces within the rachis, rachilla, and pericarp followed by inter- and intracellular invasion of the floral axis up to the testa (seed coat). Mycelia are then able to penetrate the testa allowing access to the embryo. Conidia are also formed in air spaces within the rachis allowing for the possibility of additional overwintering capability of the fungus (Smart et al. 1990).

Colonization of the embryo has also been found to occur prior to the colonization of the endosperm (Brown et al. 1995; Keller et al. 1994). Subsequently, aflatoxin has been found to be produced in higher concentrations in the embryonic and aleurone tissues of the maize kernels up until germination. Following germination, aflatoxin is no longer produced in the embryo but rather in the endosperm which is invaded by mycelia through the aleurone or scutellum (Keller et al. 1994). A likely explanation for the higher degree of aflatoxin production in embryonic tissue rather than endosperm tissue is the higher concentration of lipids found in the embryo (31% lipids) than in the endosperm (< 1% lipids) (Earle et al. 1946; Brodhagen and Keller, 2006).

2.3 Virulence Proteins and Maize Defenses

Upon successful colonization, *A. flavus* begins to employ several hydrolytic enzymes to break down the maize tissue. Such hydrolytic enzymes include amylases (α-amylase), cellulases, chitinases, cutinases, lipases, pectinases (P2c), proteases such as alkaline protease, and xylanases (Brown et al. 2001; Chen et al. 1998, 1999, 2009; Cleveland and Cotty, 1991; Cleveland et al. 2004; Fakhoury and Woloshuk, 1999; Mellon et al. 2000; Pechanova et al. 2013). These proteins facilitate nutrient uptake for fungal metabolism. These enzymes are also targets of constitutive and inducible defenses in maize. For example, α-amylase produced by *A. flavus* to breakdown complex carbohydrates and starches found in the kernel tissue has been found to be inactivated by a 14-kDa trypsin inhibitor produced in maize kernels (Chen et al. 1998). Several other defense proteins have been shown to be produced in response to *A. flavus* infection of healthy kernel tissues including β-1,3-glucanases and chitinases, which have been shown to function in the lysis of hyphal tip cells to halt fungal growth, zeamatin, and ribosome inactivating proteins (Guo et al. 1997; Huynh et al. 1992; Lozovaya et al. 1998; Mauch et al. 1988; Walsh et al. 1991).

It is also noteworthy that the successful colonization of maize tissues by *A. flavus* along with significant aflatoxin accumulation generally occurs during stress conditions, particularly drought and heat stress conditions, and contributes to the classification of *A. flavus* as an opportunistic pathogen (Payne 1998). Specifically, drought stress conditions have been shown to negatively affect the expression of genes coding for some resistance associated proteins possibly resulting in reduced accumulation of these proteins in mature maize kernels (Fountain et al. 2010; Guo et al. 2008; Scully et al. 2009; Wang et al. 2008).

Some of the proteins produced in response to stress conditions have been shown to be involved in resistance. In a series of proteomics studies by Chen et al. (2002), by comparing the

protein profiles of susceptible and resistant maize germplasm, a number of proteins related to a variety of stresses were differentially expressed. These proteins included storage proteins such as globulin 1 and globulin 2, proteins involved in drought and desiccation stress such as late embryogenesis abundant proteins (LEA 3 and LEA 14), water and osmo/salt-stress related proteins such as WSI18 and aldose reductase, and heat stress related proteins such as HSP16.9 (Chen et al. 2004). Also, Pechanova et al. (2011) examined the proteome of resistant and susceptible maize rachis during maturation and in response to infection by *A. flavus*. They found that resistant varieties accumulated high levels of abiotic stress mediating proteins, such as heat shock proteins, and antioxidant proteins, such as APx1-cytosolic ascorbate peroxidase and superoxide dismutase ([Cu-Zn]-4 and -4AP), early in development and accumulated pathogenesis-related proteins to an elevated level over time indicating that abiotic stress tolerance may play an important role in resistance to *A. flavus*.

In later studies, other proteins were also found to be up-regulated by *A. flavus* infection including glyoxalase I (GLX-I) and *Zea mays* pathogenesis-related proteins 10 and 10.1 (*Zm*PR10 and *Zm*PR10.1) (Chen et al. 2004, 2006, 2010; Xie et al. 2010). The proteins *Zm*PR10 and *Zm*PR10.1 are of particular interest as they have been shown to be inducible by both biotic and abiotic sources including pathogen infection, salicylic acid (SA) or H_2O_2 treatment of plants, or wounding (Xie et al. 2010). In pepper (*Capsicum annum*), PR10 has also been shown to be involved in programmed cell death by forming a complex with leucine-rich repeat protein1 (LRR1), (Choi et al. 2012). The amino acid sequences of both *Zm*PR10 (AAY29574.1) and *Zm*PR10.1 (ADA68331.1) also revealed that they both possess PYR/PYL/RCAR-like domains (Marchler-Bauer et al. 2013) which, like the closest homologs of these proteins in *A. thaliana*,

function in abscisic acid (ABA) signal transduction (Melcher et al. 2010; Nishimura et al. 2010; Santiago et al. 2009; Yin et al. 2009).

2.4 Systemic Plant Defense Regulation

Recent research has focused on the biological pathways involved in inducing the expression of such defense genes in plants in response to pathogen infection. Many defense genes have been found to be expressed through the process of systemic acquired resistance (SAR) which can be regulated through many chemical messengers in plants including auxins (e.g. indole-3-acetic acid, IAA), azeliac acid, dehydroabietinal, ethylene, (E)-2-hexenal, (Z)-3-hexenal, jasmonic acid (JA), salicylic acid (SA), reactive oxygen species, and lipid-based signal molecules (Durrant and Dong, 2004; Shah, 2009; van Loon et al. 2006).

Different classes of plant pathogens elicit defense signaling through different chemical messengers. Of particular interest are biotrophic and necrotrophic pathogens. Biotrophic pathogens are classified as those that survive by absorbing nutrients from living host tissue and secretes elicitors to suppress host cell death while necrotrophic pathogens, secrete elicitors into the host resulting in localized cell death, after which the pathogen acquires nutrients from the necrotic tissue (Glazebrook, 2005; Spoel et al. 2007). For example, biotrophic plant pathogens such as *Peronospora parasitica* have been found to induce SA-responsive defense gene expression in *Arabidopsis thaliana* (Glazebrook, 2005; Thomma et al. 1998), while necrotrophic plant pathogens such as *Botrytis cinerea* have also been found to induce JA- and ethylene-responsive defense gene expression in *A. thaliana* (Birkenbihl et al. 2012; Birkenbihl and Somssich, 2011; Glazebrook, 2005).

Salicylic acid (SA) has been shown to play a predominant role in the global activation of SAR in response to the infection of several pathogens throughout many plant species (Durrant

and Dong, 2004). SA is transported throughout the plant in a methylated form, methyl salicylate, through the phloem before being hydrolyzed in systemic tissues resulting in SA accumulation leading to heightened resistance, such as in the case of *Tobacco mosaic virus* (TMV) infection of tobacco (Park et al. 2009; Shah, 2009). Mechanisms related to the production of SA in response to pathogen infection, as well as the transduction of such signals to directly induce the expression of defense genes or indirectly through transcription factors to regulate defense gene expression, have been the focus of recent studies (Van Verk et al. 2011; Wang et al. 2005).

One of the primary signaling proteins identified for the induction of pathogenesis-related (PR) gene expression is the non-expressor of pathogenesis-related genes 1 (NPR 1) (Pieterse and Van Loon, 2004). NPR1 has been shown to be directly involved in SA induced defense responses through the use of mutant *A. thaliana* plants with a single recessive mutation of *npr1* resulting in insensitivity to SA, leading to highly increased susceptibility to pathogen infection, and greatly reduced expression of PR genes (Cao et al. 1994; Shah et al. 1997). NPR1 is translocated across the nuclear membrane due to the accumulation of SA in the cytoplasm. Upon entering the nucleus, NPR1 binds to members of the transcription factor (TGA) subclass of the basic leucine zipper (bZIP) family of proteins, specifically TGA2 and TGA3. These transcription factors bind to DNA at SA-responsive promoter regions (TGACG), which results in the expression of PR genes such as PR-1 (Pieterse and Van Loon, 2004; Spoel et al. 2009; Zhou et al. 2000). Recent studies have also shown that the regulation of NPR1 levels in the nucleus is mediated by the ubiquitinylation of NPR1 by the Cullin3-based E3 ubiquitin ligase (CUL3), with the NPR1 paralogues NPR3 and NPR4 serving as adaptors, and subsequently degraded by the 26S proteosome (Fu et al. 2012; Pintard et al. 2004; Spoel et al. 2009). This process is necessary

for maintaining and preventing pre-mature activation of NPR1 mediated SAR responses (Fu et al. 2012; Spoel et al. 2009)

2.5 WRKY Transcription Factors and Their Regulation

NPR1/TGA has also been found to promote the expression of additional transcription factors involved in resistance to pathogen infections, such as WRKY transcription factors (Wang et al. 2006). WRKY transcription factors, first described by Ishiguro and Nakamura (1994), represent a complex network of defense gene expression regulators. These transcription factors are characterized by their distinctive structural features and DNA binding affinity. The WRKY transcription factor DNA binding domain consists of approximately 60 residues with an almost invariant amino acid sequence of WRKY at the N-terminus of the domain, and a zinc finger structure at the C-terminus (Rushton et al. 1996, 2010). This domain binds highly conserved promoter regions referred to as W-boxes with sequence of C/TTGACC/T (Euglem and Somssich, 2007). Based on key protein structural features and sequence analysis, WRKY transcription factors can be grouped into Group I, IIa, IIb, IIc, IId, IIe, and III classifications with not all Group II subgroups being monophyletic (Chen et al. 2012b; Rushton et al. 2010; Zhang and Wang, 2005).

The expression of WRKY transcription factors can be influenced by a wide range of defense-related pathways including SA response through NPR1/TGA (Euglem and Somssich, 2007). In addition to this, mitogen-activated protein kinase (MAP kinase; MAPK) cascades resulting from pathogen recognition by pathogen associated molecular pattern (PAMP) receptors leading to PAMP-triggered immunity (PTI) can also play a role in regulating WRKY transcription factor expression. PTI is initiated by the binding of PAMPs to receptor-like kinase (RLK) proteins which results in the initiation of a signaling cascade through phosphorylation

from MAP kinase kinase kinase (MAP3K) to MAP kinase kinase (MAP2K), and finally to MAPK (Chisholm et al. 2006). For some group I WRKY transcription factors, such as *At*WRKY25 and *At*WRKY33, the signals are then passed to the nuclear localized coupling factor MKS1 which promotes the expression of the target gene(s) (Andreasson et al. 2005; Euglem and Somssich, 2007; Li et al. 2011; Qiu et al. 2008; Rushton et al. 2010). WRKY transcription factor expression can also be modulated by effector triggered immunity (ETI) in which recognizing avirulence effector proteins secreted by pathogens into the plant cell results in signaling cascades to initiate defense gene expression. For example, resistance to barley powdery mildew (*Blumeria graminis* f.sp. *hordei*), requires the recognition of the avirulence effector protein AVR10 by the defense protein mildew-resistance locus A (MLA). The association of MLA with *Hv*WRKY1 and *Hv*WRKY2 then functions to initiate ETI (Euglem and Somssich, 2007; Padney and Somssich, 2009; Shen et al. 2007; Rushton et al. 2010).

Several WRKY transcription factors (TF) have been shown to have marked effects on the expression of genes associated with defense. For example, in *Arabidopsis*, following SA-concentration dependent nuclear translocation of NPR1 as previously described, NPR1 binds to TGA transcription factors resulting in the expression of numerous WRKY TFs capable of either promoting or repressing defense-related gene expression including *At*WRKY18, -38, -53, -54, -58, -59, -66, and -70 (Wang et al. 2006; Euglem and Somssich, 2007). In particular, *At*WRKY70 has been shown to be involved in the expression of SA-responsive defense related genes including PR1, PR2, and PR5 that are involved in SAR. In addition, *At*WRKY70 has been shown to function in crosstalk between SA and JA pathways in a concentration-based manner. Elevated levels of *At*WRKY70 tend to suppress JA-responsive genes and promote SA-responsive genes. Conversely, reduced levels of *At*WRKY70 tend to promote JA-responsive genes and suppress

SA-responsive genes (Li et al. 2004). Also, *At*WRKY58 has been shown to assist in the regulation of SAR by acting as a negative regulator of resistance at SA levels below a certain threshold. This characteristic may lend to prevention of unwarranted activation of SAR defenses as well as the deactivation of SAR once the pathogen challenge has subsided (Wang et al. 2006).

Another group of examples includes several key WRKY TFs regulated by a MAP-kinase cascade triggered during PTI as previously described such as, *At*WRKY28, *At*WRKY33, *At*WRKY46, *At*WRKY53, and parsley *Pc*WRKY1. *At*WRKY28 and *At*WRKY46 regulate the expression of genes coding for isochorismate synthase (ICS1) and AVR_{PPHB} Susceptible 3 (PBS3), respectively. These two enzymes function in the production of SA utilized in NPR1/TGA mediated SAR (Euglem and Somssich, 2007; Van Verk et al. 2011). *At*WRKY33 is negatively regulated by MPK4 when it is bound to the complex of the *At*WRKY33 protein and MKS1 (Andreasson et al. 2005; Eulgem and Somssich, 2007; Ishihama and Yoshioka, 2012; Qiu et al. 2008; Rushton et al. 2010). In addition, MPK3 and MPK6 have been found to interact with *At*WRKY33 and phosphorylate the protein in response to pathogen infection (Ishihama and Yoshioka, 2012; Mao et al. 2011). In addition to NPR1/TGA, *At*WRKY53 can also be regulated through MAPK pathway components, specifically MEKK1 (Eulgem and Somssich, 2007; Miao et al. 2004, 2007; Rushton et al. 2010). Finally, *Pc*WRKY1 has been shown to function in autoregulation as well as in the transcriptional activation of *PcPR10* expression (Ülker and Somssich, 2004).

Given the high degree of association of WRKY TFs with key SA-based defense pathways such as SAR, it is possible that key maize defense genes and proteins may be regulated directly by W-box promoter elements, or indirectly through regulation of SA-responsive defense pathway elements. However, a recent review discussing the role of NPR1 in maize indicated that

the knockout or over-expression of the maize ortholog of *A. thaliana* NPR1 resulted in no change in disease responses (Balint-Kurti and Johal, 2009; Johal et al. Unpublished). Conversely, studies by Chern et al. (2001, 2005) have shown that NPR1 in rice functions similarly to that described for *A. thaliana* and that overexpression of rice NPR1 results in constitutive activation of defense responses. Therefore, since transformants involving rice NPR1 modulate host resistance while those using maize NPR1 do not, it is possible that mechanisms and pathways involved in induced defense responses may vary between monocot species (Balint-Kurti and Johal, 2009), but further studies are needed to identify specific causes for any functional dissimilarity.

Host defenses mediated by jasmonic acid and ethylene can also be modulated by WRKY transcription factors. In a recent series of studies by Birkenbihl and Somssich (2011) and Birkenbihl et al. (2012), it was clearly demonstrated that *At*WRKY33 is required for the induction of JA-promoted defense genes such as defensins (PDF1.1 and PDF1.2). Aspects of JA biosynthesis and defenses can also be influenced by WRKY transcription factors, such as *At*WRKY11, -17, -50, and -51. *At*WRKY11 and *At*WRKY17 are functionally redundant and serve to promote JA biosynthesis genes, such as lipoxygenase 2 (LOX2) and allene oxide synthase (AOS), while simultaneously suppressing *At*WRKY70, which, as previously discussed, promotes SA-induced basal defenses (Journot-Catalino et al. 2006). *At*WRKY17 may also function in bacterial pathogen resistance; however, a recent study found that silencing *At*WRKY17 increases *A. thaliana* susceptibility to *Agrobacterium tumefaciens* (Lacroix and Citovsky, 2013). In addition, reduced levels of oleic acid (18:1), a biological precursor to JA (Heldt and Piechulla, 2010; León and Sánchez-Serrano, 1999), results in the induction of *At*WRKY50 and *At*WRKY51 which function to suppress JA-based defenses (e.g. PDF1.2) and promote SA-based defense genes (Gao et al. 2011). Through a MPK3/6 signal transduction

pathway, *At*WRKY33 has also been shown to promote the expression of 1-amino-cyclopropane-1-carboxylic acid (ACC) synthase genes ACS2 and ACS6, enzymes involved in ethylene biosynthesis (Li et al. 2012).

WRKY transcription factors have also been found to be involved in plant responses to environmental stresses. Responses to abiotic stresses such as heat stress and salt stress have been shown to be mediated by pathways under the regulation of WRKY transcription factors (Chen et al. 2012b). For example, *At*WRKY25, -26, -33, and -39 have been shown to promote thermotolerance under heat stress conditions (Li et al. 2010, 2011). Functional orthologs of *At*WRKY33 in other species have also been shown to be involved in abiotic stress responses such as its homolog in maize (annotated as *Zm*WRKY53 in the Genebank and in this study), which enhances salt stress tolerance when overexpressed transiently in *A. thaliana* and is induced by drought, salt, and cold stress as well as by ABA application (Li et al. 2013). Abiotic stress responses can be mediated by interactions between ABA and calcium signaling through calmodulins (Hu et al. 2006; Reddy et al. 2011). Calmodulins have been found to bind to several transcription factors including TGA3 and TGA6, as well as several WRKYs including *At*WRKY7, -21, -43, -45, -50, and -53 (Chi et al. 2013; Popescu et al. 2007). Given these findings, it is probable that the orthologs of some of these WRKY transcription factors in maize serve dual roles and are involved not only in pathogen defense signaling, but also in abiotic stress response. This has recently been demonstrated to be the case in drought stress responses (Wei et al. 2012).

2.6 Present Study

Some maize defense genes have been shown to be up-regulated by phytohormone application. Examples of this are *ZmPR10* and *ZmPR10.1*, which were up-regulated in response

to applied SA and in response to *A. flavus* infection (Chen et al. 2006, 2010; Xie et al. 2010). *Zm*PR10 and *Zm*PR10.1, both of which possess anti-fungal and RNase activities and function in maize resistance to *A. flavus* infection, have also been found to possess W-box elements in their promoter regions strongly indicating the possible involvement of WRKY transcription factors in regulating the expression of these genes (Chen et al. 2006; Liu and Ekramoddoullah, 2006; Xie et al. 2010; Xie et al. unpublished). However, no studies have confirmed the involvement of SAR in maize defense to *A. flavus* infection or aflatoxin accumulation. Therefore, in order to better understand the mechanism of defense gene induction in maize in response to *A. flavus* infection, transcriptional regulators, such as WRKY TFs, need to be examined. In addition, recent microarray analyses of a resistant and a susceptible maize line derived from a cross between 1638 and GT-MAS:gk, Eyl25 and Eyl31, respectively (Menkir et al. 2006, 2008), revealed varying levels of expression of several WRKY TFs between the two lines. This indicates that WRKY TFs may play an important role in coordinating transcriptional reprogramming for defense responses in maize to *A. flavus* infection and/or aflatoxin contamination (Luo et al. 2011).

In this study, therefore, we examined available microarray data for possible candidate WRKY TFs involved in resistance to *A. flavus* colonization of maize kernels, and compared the expression of these candidate WRKY genes in resistant and susceptible maize lines in response to *A. flavus* inoculation under field conditions using real-time/quantitative polymerase chain reaction (qPCR) techniques. In addition, the expression of several indicator genes was used to determine which major defense pathways, SA or JA/Ethylene, are used in conjunction with WRKY transcription factors to regulate maize defense to *A. flavus* infection, specifically the maize homologs of NPR1 and PR-1, which regulate SA-based defenses (Spoel et al. 2007; Zhang

et al. 1999), and ethylene responsive factor1 (ERF1), which regulates JA and ethylene-based defenses (Lorenzo et al. 2003). In addition, expression variation over time was examined in order to reveal possible variations in WRKY TF expression over the course of *A. flavus* infection. The identification of maize WRKY TFs specifically induced or repressed by *A. flavus* infection and subsequent aflatoxin accumulation with homologies to WRKY TFs in other plant species, such as rice (*Oryza sativa*) or *A. thaliana*, will allow for a better understanding of the possible maize defense mechanisms involved in producing the resistance phenotype. In addition, the identification of specific maize WRKY TFs regulated by *A. flavus* infection may allow for their use in marker assisted selection (MAS) in breeding applications.

3. MATERIALS AND METHODS

3.1 WRKY Transcription Factor Identification and Selection

Maize putative WRKY transcription factor sequences, 202 in total, were obtained from the Plant Transcription Factor Database v3.0 (Pérez-Rodríguez et al. 2010). In order to determine which of these many putative WRKY TFs to examine in this study, the sequences obtained from the database were then searched using the National Center for Biotechnology Information (NCBI) Basic Local Alignment Search Tool (BLAST) for the database of the Maize Oligonucleotide Microarray Project (http://www.maizearray.org). Having identified the available WRKY transcription factors available on the array and their correlating annotations (Maize Array Annotations v.1.0), we examined the results of a previously performed microarray study by Luo et al. (2011; Unpublished data) in which the expression profiles in kernels of maize genotypes Eyl25 (Resistant) and Eyl31 (Susceptible) upon infection with *A. flavus* in kernel screening assay (KSA) conditions described by Brown et al. (1995). Maize WRKY TF annotations showing significant up- or down-regulation by *A. flavus* infection were selected for examination in the present study (Table 3.1). In addition, a maize WRKY TF, PTZm631 (NM_001196138.1), which was found to be significantly induced by *A. flavus* infection in a susceptible commercial hybrid N83-N5 (Syngenta, Basel, Switzerland) in immature kernel tissue, by Han et al. (2010, Unpublished data) was also included in the present study.

3.2 Plant Materials

In 2010, 2011, and 2012, two inbred maize lines, B73 and TZAR101, were grown in field conditions with adequate irrigation at the Burden Research Center, Baton Rouge, LA, USA. B73 is a standard inbred line which is known to be susceptible to *A. flavus* colonization and aflatoxin accumulation (Chen et al. 1998; Scully et al. 2009). TZAR101 is a relatively new inbred line

Table 3.1 Prospective maize WRKY transcription factor annotations identified from maize microarray and quantitative/real-time PCR.

		Fold Change[2]	
Annotation	**Putative gene function[1]**	**Eyl25[3]**	**Eyl31[4]**
MZ00019797	Putative WRKY transcription factor, *Oryza sativa* (japonica cultivar-group)	N/A	13.78
MZ00021479	Putative WRKY transcription factor, *Oryza sativa* (japonica cultivar-group)	1.2	1.7
MZ00026377	WRKY1, *Zea mays*	1.5	2.62
MZ00042391	N/A, *Oryza sativa* (japonica cultivar-group)	1.7	3.1
MZ00042508	Putative WRKY transcription factor, *Oryza sativa* (japonica cultivar-group)	N/A	3.7

[1]Putative gene function obtained from maize array annotation v4.0; 58k maize microarray

[2]Fold change in gene expression shown by qPCR during *A. flavus* infection in comparison to non-treated controls (Luo et al. 2011, Luo et al. Unpublished data). N/A indicates no detectible variation in expression.

[3]Resistant variety

[4]Susceptible variety

developed by the United States Department of Agriculture – Agricultural Research Service (USDA-ARS) and the International Institute of Tropical Agriculture (IITA) which is resistant to *A. flavus* colonization and aflatoxin accumulation (Menkir et al. 2008).

In each year, plots of each variety were planted in a randomized block design along with other IITA lines and inbreds utilized in concurrently running experiments. In 2010, two plots of B73 and one plot of TZAR101 were grown to serve as two and one biological replicates respectively. In 2011 and 2012, one plot of B73 and two plots of TZAR101 were grown and serve as one and two biological replicates, respectively. This variation in plot numbers is due to seed availability in each year. This study, therefore, consists of at least four biological replicates for each line over three years. All ears used in this study were self-pollinated in order to ensure homozygosity in the kernel tissues used for expression analysis as follows. Immature ear shoots were covered prior to anthesis with a no. 217 shoot bag (Lawson, Northfield, IL, USA). Upon

anthesis and the emergence of fully developed anthers on the tassel, the entire tassel was covered with a no. 404 tassel bag (Lawson) and secured with paper clips. The following day, pollen was transferred to the emerged silk using the tassel bag and placing the bag around the emerged ear. The shoot bag was discarded during the process and the tassel bag was left in place and secured to the plant to prevent contamination with additional pollen. Tassel bags were labeled using a permanent marker with the pollination date to facilitate sample collection over a time course.

3.3 Inoculation and Sample Collection

At 14 days after pollination (DAP), the experimental treatments were applied. For inoculated samples, the maize ears were inoculated with 5.0 mL per ear (divided evenly across four injection sites) of *A. flavus* (Strain AF13; ATCC 96044, SRRC 1273) conidial suspension (4×10^6 conidia/mL in 0.01% (*v*/*v*) tween 20) directly through husks using a 60.0-mL hypodermic syringe with an 18-gage hypodermic needle. To simulate the physical damage resulting from inoculation, wounded control samples were stabbed with a sterile 18-gage hypodermic needle which was used to wound multiple plants without additional sterilization while having no contact with *A. flavus* inoculum. Remaining ears in the plot served as non-treated controls. Self-pollinated ears were then collected over a time course from 0 to 18 days after inoculation (DAI).

In 2010, two replicate samples were collected at 1, 2, 4, 6, 10, 14, and 18 DAI from each treatment, with the exception of wounded controls, which were collected up to 10 DAI. In 2011, two replicate samples were collected at 0, 1, 3, 6, 10, 14, and 18 DAI from each treatment, with the exception of wounded controls, which were collected up to 6 DAI. In 2012, three replicate samples were collected at 0, 1, 4, 6, 10, 14, and 18 DAI from each treatment with the exception of non-treated controls which were not collected. Changes in sample collection timing and

treatment applications were due to limited availability of plants in some plots as well as preliminary data suggesting that gene expression variations in response to wounding tend to occur before 6 to 10 DAI. All samples were collected, stored on ice and transported to the laboratory. Kernels (12-16, approx. 5g) surrounding the inoculation or wounding sites on treated ears were then removed from the ears and placed into 50-ml conical tubes and flash-frozen in liquid N_2. The samples were then homogenized into a fine powder using a chilled mortar and pestle, and stored at -80°C until further use. This homogenized tissue was then used for nucleic acid extractions in preparation for expression analyses using quantitative/real-time polymerase chain reaction (qPCR).

3.4 Field-Based Assay for Aflatoxin Accumulation Resistance

In addition to plants inoculated for use in qPCR applications, additional ears for both B73 and TZAR101 for each treatment were collected at full maturity (~ 60 DAP, seed moisture level < 15%) for aflatoxin analysis in order to confirm their resistance under Louisiana field conditions in 2012. In each biological replicate, up to 10 plants each were used as non-treated controls, wounded, and inoculated treatments. Kernels surrounding the inoculation sites, approximately 20g, were removed and sent to Dr. Robert L. Brown's laboratory at the Southern Regional Research Center (SRRC), USDA-ARS in New Orleans, LA, for aflatoxin analysis using an AgraQuant total aflatoxin ELISA test kit (Romer Labs, Union, MO, USA) according to the manufacturer's instructions (Zheng et al. 2005).

3.5 Total RNA Isolation and Complementary DNA Synthesis

Total RNA was extracted from 100 mg of homogenized kernel tissue from each sample using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Isolated total RNA was then treated with DNase (Qiagen, Valencia, CA, USA). In

addition, given the high concentration of carbohydrates within the immature maize kernel tissue, an additional centrifugation step of three minutes at 12,000 x g was included following DNase digestion in order to remove any un-dissolved starch from the solution. The supernatant was then transferred to a separate 1.5mL microfuge tube and purified using a cleanup column from an RNeasy Plant Mini Kit (Qiagen) according to the manufacturer's instructions. The quantity of purified total RNA were estimated based absorbance values at 230, 260, and 280 nm measured using a Nano-Drop ND-1000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA) (Fountain et al. 2010).

Complimentary DNA (cDNA) was then synthesized from the isolated total RNA using a TaqMan Reverse Transcription Reagents Kit (Applied Biosystems, Warrington, UK) according to the manufacturer's instructions. For reverse transcription, a 20.0 µl reaction volume contained the following components and final concentrations of: 25 ng/µl total RNA, 1.0 X TaqMan RT buffer, 5.5 mM $MgCl_2$, 500 µM for each dNTP, 2.5 µM random hexamers, 0.4 U/µl RNase inhibitor, and 1.25 U/µl MultiScribe Reverse Transcriptase. The reverse transcription reaction was performed using a MyCycler Thermal Cycler (Biorad, Hercules, CA, USA) with the following cycling parameters: 25°C for 10 min, 48°C for 30 min, and 95°C for 5 min. The synthesized cDNA was then stored at -20°C until use in qPCR.

3.6 Primer Design and Amplification Efficiency Analysis

The cDNA sequences of the candidate WRKY genes were retrieved from Genebank and analyzed using Beacon Designer Software (Biorad) to design gene specific qPCR primers with an amplicon size of 80 – 150 bp and a melting temperature (T_m) of 60°C ± 1°C. In order to prevent off-target amplification, BLAST was used to identify the ten most homologous sequences to the WRKY TF cDNA sequence. These sequences were then aligned using

ClustalW2 (EMBL-EBI; http://www.ebi.ac.uk/Tools/msa/clustalw2/). Primer suggestions from the Beacon Designer software were then compared to the alignments and those primer pairs that possessed the least homology to off-target sequences, particularly at the 3' end of the primer sequences, were selected.

The amplification efficiency for each set of real time PCR primers was determined using serial diluted genomic DNA extracted from newly emerged leaf tissue of a commercial hybrid, N83-N5 (Syngenta), isolated using a GenElute Plant Genomic DNA Miniprep Kit (Sigma Aldrich, St. Louis, MO, USA) according to the manufacturer's instructions. If primer dimerization was observed, corresponding cDNA was used instead to determine whether this could resolve the primer dimerization issue. In cases when primer dimerization levels or amplification efficiency was not ideal, a new set of primers were designed. A concentration gradient of five, ten-fold serial dilutions from 110 ng to 11 pg was used in the experiment. For the primers of the SA and JA/ethylene-based defense pathway indicator genes (*ZmNPR1*, *ZmPR-1*, and *ZmERF1*), a concentration gradient of five-fold serial dilutions from 29 ng to 46.4 pg was used for amplification efficiency determination.

For amplification efficiency analysis, qPCR was performed in a 25 µl reaction volume containing the following: 1X SYBR Green PCR Master Mix (Applied Biosystems), 0.4 µM forward primer, 0.4 µM reverse primer, and template from the dilution series with the previously indicated concentrations. Three technical replicates were included for each sample. qPCR was performed in an ABI Prism 7000 Sequence Detection System (Applied Biosystems) using the following parameters: 50°C for 2 min, 95°C for 10 min, and 40 amplification cycles of 95°C for 15 sec, and 60°C for 1 min. Dissociation curve analysis was performed at the end of the amplification cycles to examine for the occurrence of primer dimerization. Standard curve

analysis resulting from plotting observed cycle threshold (C_T) values versus Log(concentration) followed by linear regression analysis was then used in conjunction with the equation Efficiency = $10^{(-1/Slope)} - 1$ (Pfaffl, 2001) to calculate amplification efficiency for each primer pair. Primers with amplification efficiency of >87% (E+1 = 1.87) are used in this study (Pfaffl, 2004). A full list of the primer sequences used in this study, their corresponding amplification efficiencies, and the database accessions for each gene can be found in Table 3.2.

3.7 Gene Expression Analysis

The expression levels of the selected maize WRKY TF and pathway component genes in each line in response to each treatment was analyzed through qPCR using the following setup in a 15µl reaction volume: 1X SYBR Green PCR Master Mix (Applied Biosystems), 0.4 µM forward primer, 0.4 µM reverse primer, and 25 ng template cDNA. Three technical replicates were done for each sample. qPCR was then performed using an ABI Prism 7000 Sequence Detection System (Applied Biosystems) with cycling parameters: 50°C for 2 min, 95°C for 10 min, and 40 amplification cycles of 95°C for 15 sec, and 60°C for 1 min. Dissociation curve analysis was performed at the end of the amplification cycles to test for primer dimerization. Threshold cycle (Ct) values were then calculated using a fluorescence threshold of 0.3 with a baseline automatically calculated by the equipment software. Relative gene expression levels were then determined by comparing the Ct of the target gene to that of the internal reference gene, *Zm*18S rRNA, using the following equation: relative gene expression = $[(E+1)^{(Ct\ Zm18S\ rRNA)} / (E+1)^{(Ct\ target\ gene)}]$, where E is the target gene primer amplification efficiency derived from equation Efficiency = $10^{(-1/Slope)} - 1$ (Chen et al. 2010; Jiang et al. 2012; Pfaffl, 2001).

Table 3.2 Gene accession numbers and primer sequences for qPCR analysis.

Gene ID	Microarray Accession	Plant TFD Accession	Genebank Accession*	Primer Sequences		$M_{Reg.}$	E(%)
***Zm* WRKY19**	MZ00019797	GRMZM2G063880_P01	AFW81188.1	*Zm* WRKY-F	5'-CGCCGACTTCCCGCTCTTC-3'	-3.41	0.96
				Zm WRKY-R	5'-CGAACTCCACGCCGATGCC-3'		
***Zm* WRKY21**	MZ00026377	GRMZM2G117394_P01	NP_001150830.1	*Zm* WRKY21-F	5'-CGCTCGTCGTCACCACCAC-3'	-3.22	1.05
				Zm WRKY21-R	5'-CTCTCCACTGCCGTCCTGTTG-3'		
***Zm* WRKY53**	MZ00021479	GRMZM2G012724_P01	NP_001147949.1	*Zm* WRKY53.1-F	5'-GCGGCGACTTCTCCTTCCAC-3'	-3.32	1.00
				Zm WRKY53.1-R	5'-CACTTGCTGCTCTTGCTCCTTG-3'		
***Zm* WRKY53.1**	MZ00042508	GRMZM2G150441_P03	NP_001147551.1	*Zm* WRKY53-F	5'-CCACACGGCTACCTCCAACG-3'	-3.33	1.00
				Zm WRKY53-R	5'-CTTGCTGCTCTGCTCCTCCTC-3'		
***Zm* WRKY67**	PTZm631**	GRMZM2G076878_P01	NP_001148599.1	*Zm* WRKY67-F	5'-TCAGAACGAGATCAGAGGTGGA-3'	-3.16	1.07
				Zm WRKY67-R	5'-TCTTGACAGCCTTCTTGCCAT -3'		
***Zm* WRKY68**	MZ00042391	GRMZM2G071907_P01	ACG45417.1	*Zm* WRKY68-F	5'-TAGAAAGAGGGAGAGGAGGACACC-3'	-3.24	1.04
				Zm WRKY68-R	5'-AGCCTGAGCGAGCACCAATC-3'		
***Zm* ERF-1**	·	·	NP_001105270.1	*Zm* ERF1-F	5'-TCGTCCTAGTCGGTGTGATGTGAT-3'	-3.12	1.09
				Zm ERF1-R	5'-TCTTGATTCCTCATCAACTTGCGAGT-3'		
***Zm* NPR-1**	·	·	ACG45791.1	*Zm* NPR1-F	5'-TCGCGCTTGCAGATGTCAATCA-3'	-3.55	0.91
				Zm NPR1-R	5'-TCAAATGTGAGGTCTGATGGCCGA-3'		
***Zm* PR-1**	·	·	NP_001152581.1	*Zm* PR1-F	5'-GCCACTACACCCAGATCATG-3'	-3.67	0.87
				Zm PR1-R	5'-AAAGGAACGGTGTCAGTACG-3'		
***Zm* 18S**	·	·	AF168884.1	*Zm* 18S-F	5'-GAGAAACGGCTACCACATCCA-3'	-3.11	1.10
				Zm 18S-R	5'-ACGCGCCCGGTATTGTTAT-3'		

$M_{Reg.}$ = Slope of linear regression in standard curve analysis.

E(%) = Amplification efficiency calculated from the equation $E = 10^{(-1/Slope)} - 1$

*NCBI Genebank accession of gene product. Protein amino acid sequences for WRKY transcription factors obtained by BLASTp of PlantTFD sequences.

**From Han et al. 2010

3.8 Statistical Analysis

The relative expression data from the three years of the study were combined for analysis for all available time points and treatments. The relative expression levels were transformed by taking the natural logarithm of the relative expression levels after they had been multiplied by 1.0×10^9 to allow for the absence of negative values in the data set. Log transformation was used to compensate for variation among the yearly replications of the experiment. Examination of significant interactions between fixed effects and the determination of averages and standard error of the three-way interactions of variety, treatment, and time point were done using a factorial analysis of variance (ANOVA) using Proc Mixed in SAS (Statistical Analysis System, SAS Institute, Cary, NC, USA). Compensations in Proc Mixed were made to correct for any non-normal distributions using the Kenward-Rogers adjustment to the denominator degrees of freedom (Kenward and Roger, 1997). These analyses used modified marginal means to compensate for any missing points that would confound least square means calculations for type III fixed effects (Saxton, 1998; Searle et al. 1980). For aflatoxin level analysis, an ANOVA with modified marginal means in conjunction with Tukey's LSD for post-hoc analysis was used (Saxton, 1998). Significance in this study was defined by a confidence interval $\geq 95\%$ ($\alpha = 0.05$).

4. RESULTS

4.1 Aflatoxin Accumulation in Maize Lines B73 and TZAR101

The detectable aflatoxin levels in the mature kernels of the two maize lines used in this study were examined in order to evaluate their potential resistance to *Aspergillus flavus* colonization and subsequent aflatoxin contamination. In 2012, B73, the known susceptible line (Chen et al. 1998; Scully et al. 2009), showed significantly greater aflatoxin accumulation in the inoculated samples with an average aflatoxin level of 2042.5 ± 122.6 ppb (±SE; Range = 320.0 – 4200.0 ppb) compared to the resistant line, TZAR101 (Menkir et al. 2008), with an average aflatoxin level of 203.0 ± 89.8 ppb (Range = 0.0 – 650.0 ppb) in inoculated samples (Figure 4.1). As expected, the non-treated controls and wounded samples in both lines contained low levels of aflatoxin: 12.5 ± 115.2 ppb (Range = 0.0 – 79.0 ppb) and 10.9 ± 131.7 ppb (Range = 0.0 – 40.0 ppb), respectively, for B73; and 1.6 ± 86.7 ppb (Range = 0.0 – 8.3 ppb) and 4.9 ± 99.0 ppb (Range = 0.0 – 34.0 ppb), respectively, for TZAR101 (Figure 4.1). The large standard error present in the non-treated control and wounded samples is likely due to natural infection of the ears by *A. flavus* in the environment possibly through the wounding site created by the inoculation technique or insect damage.

4.2 Selection and Identification of Prospective WRKY Transcription Factor Genes

Two hundred and two (202) putative maize WRKY transcription factor sequences were obtained from the Plant Transcription Factor Database v3.0 (Pérez-Rodríguez et al. 2010). These sequences were searched using BLAST against the database of the Maize Oligonucleotide Microarray Project (http://www.maizearray.org) to identify specific annotations for the putative WRKY transcription factors in maize. These annotations were then searched in maize microarray data obtained by Luo et al. (2011; unpublished data) in which kernels of resistant and susceptible

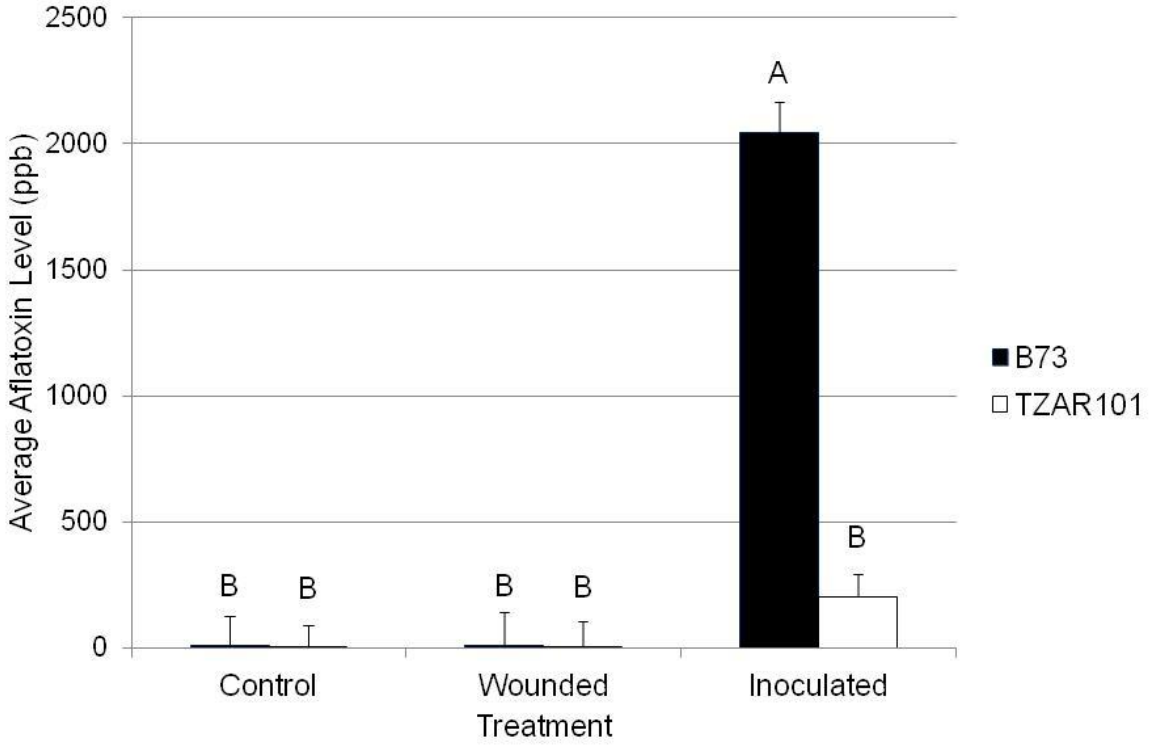

Figure 4.1 Field assay for resistance to aflatoxin accumulation in maize lines B73 and TZAR101.Aflatoxin levels were detected in analyses performed by USDA-ARS SRRC. Black bars (■) represent the average aflatoxin level in ppb for the susceptible line, B73, for the 2012 field assay in non-treated control, wounded, and inoculated samples. White bars (□) represent the average aflatoxin level in ppb for the resistant line, TZAR101, for the 2012 field assay in non-treated control, wounded, and inoculated samples. Post-hoc analysis was performed using Tukey's LSD with $\alpha = 0.05$. Error bars represent the standard error.

maize genotypes Eyl25 (R) and Eyl31 (S) were inoculated with *A. flavus* using the kernel screening assay (KSA) protocol described by Brown et al. (1995).

Comparison of the maize WRKY TF sequences from the Plant Transcription Factor Database (Pérez-Rodríguez et al. 2010) with annotations in the microarray database with the microarray data obtained by Luo et al. (2011; unpublished data) led to the identification of five candidate WRKY TFs for use in the present study. These genes can be identified by the following annotations: MZ00019797, MZ00021479, MZ00026377, MZ00042391, and MZ00042508 (Table 3.2). Of these annotations, MZ00021479, MZ00026377 and MZ00042391

were up-regulated in Eyl25 1.2, 1.5, and 1.7 fold respectively in comparison to non-treated controls of the same variety. For Eyl31, annotations MZ00019797, MZ00021479, MZ00026377, MZ00042391, and MZ00042508 were up-regulated by 13.78, 1.7, 2.62, 3.1, and 3.7 fold respectively, in comparison to non-treated controls of the same variety with the expression of MZ00019797 and MZ00042508 appearing only in this line and not in the resistant line, Eyl25. These identified WRKY TFs and their relative fold changes found by Luo et al. (2011; unpublished data) in KSA conditions are summarized in Table 3.1. In addition to these WRKY TFs identified from the microarray study, examination of data obtained by Han et al. (2010; unpublished data) yielded the identification of an additional WRKY TF, PTZm631, which was found to be significantly up-regulated, >20 fold, compared to non-treated controls in response to *A. flavus* inoculation in immature kernel tissues of the susceptible commercial hybrid N83-N5 (Syngenta). Given the degree of up-regulation of this particular WRKY TF, this gene was added to the list of candidate WRKY TFs for further examination in the present study.

The identity of the specific maize WRKY TFs the microarray accessions described were obtained by searching the corresponding amino acid sequences from the Plant Transcription Factor Database of the microarray accessions using BLASTx, a protocol which searches the amino acid sequence database for proteins using a nucleotide query sequence. MZ00019797 does not have high homology to currently characterized maize WRKY TFs, but does to an available putative WRKY TF (AFW81188.1; Coverage = 69%, ID = 46%). This putative WRKY TF is homologous to *Os*WRKY19 (DAA05084.1; Coverage = 94%, ID = 51%), therefore this putative WRKY transcription factor was annotated *Zm*WRKY19 in this study. MZ00021479 and MZ00042508 were homologous to two entries for *Zm*WRKY53, NP_001147949.1 (Coverage = 99%, ID = 64%) and NP_001147551.1 (Coverage = 60%, ID = 100%), respectively, with these

two sequences sharing only 83% homology at the protein level. This indicates that these two accessions are isoforms or distinct WRKY transcription factors and may possess different biological functions. MZ00026377 was found to be homologous to *Zm*WRKY21 (NP_001150830.1; Coverage = 65%, ID = 95%), MZ00042391 was found to be homologous to *Zm*WRKY68 (ACG45417.1; Coverage = 56%, ID = 100%), and PTZm631 from Han et al. (2010) was found to be homologous to *Zm*WRKY67 (NP_001148599.1; Coverage = 71%, ID = 97%).

4.3 Expression Analysis of WRKY Transcription Factor Genes

The expression levels of the selected maize WRKY TF genes in each line were examined for responses in immature maize kernel tissue to each treatment (non-treated control, wounded, and inoculated with *A. flavus*) over a time course ranging from 0 to 18 DAI across three years of study using qPCR. The relative expression data were combined for analysis for all available time points and treatments, and transformed as described in Materials and Methods. The transformed data were then analyzed for individual maize WRKY TFs by factorial ANOVA using a Proc Mixed procedure with SAS (SAS Institute).

The expression levels of the gene annotated MZ00019797, identified as a putative maize WRKY19 TF, were analyzed over the time course in both the susceptible B73 line (Figure 4.2; Graph A) and the resistant TZAR101 line (Figure 4.2; Graph B). In B73, this putative *ZmWRKY19* gene was found to be induced by inoculation from 0 to 6 DAI compared to non-treated control with the exception of 3 DAI, which had only one year of biological data (2011) and may be the result of environmental variation between years. The expression levels of this induction by inoculation and suppression in the non-treated control are significantly different from each other, but neither is significantly different from the expression levels of the wounded

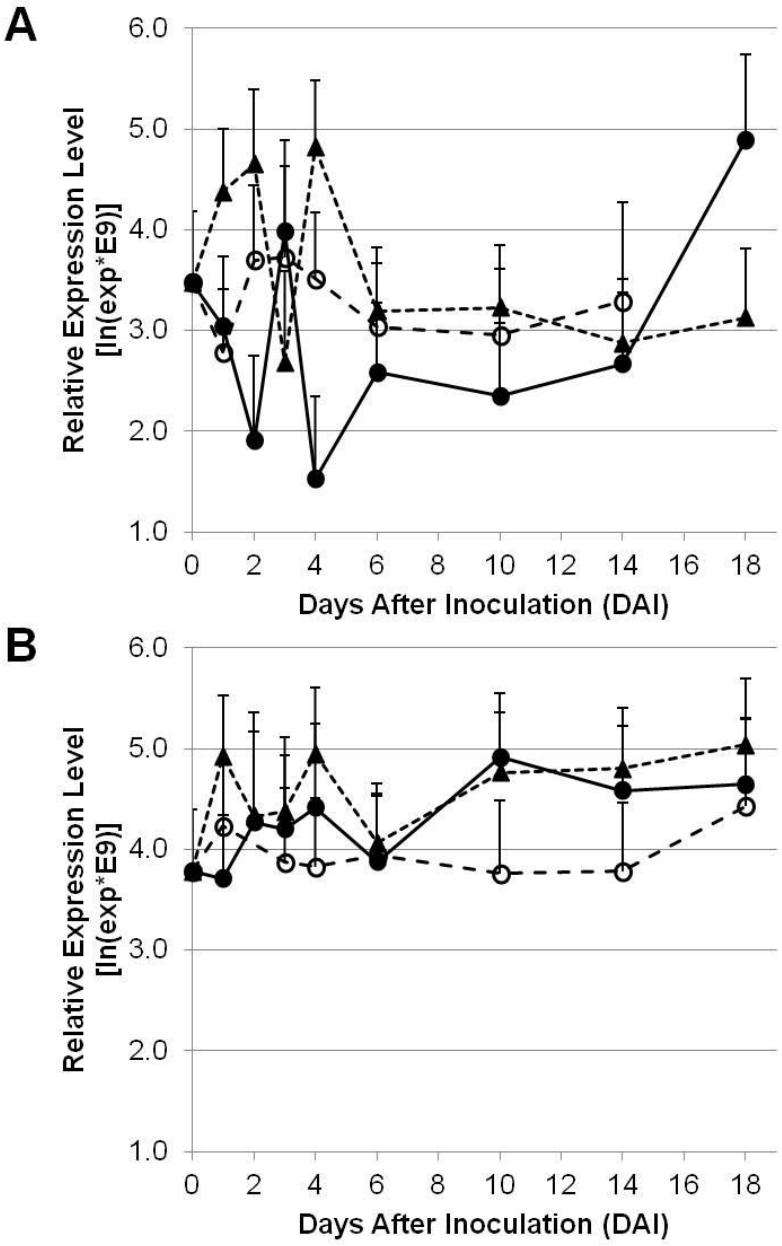

Figure 4.2 Relative expression levels of the putative *ZmWRKY19* gene (Microarray Accession: MZ00019797). Plotted values were calculated by taking the natural logarithm of the product of the relative expression of the putative *ZmWRKY19* gene by $1.0x10^9$ over the time course. Data points represent the average of the relative expression levels for each treatment and time point for all available years and plots. A. Relative expression levels over the time course in non-treated controls (●), wounded (○), and inoculated (▲) treatments for the susceptible line, B73. B. Relative expression levels over the time course in non-treated controls (●), wounded (○), and inoculated (▲) treatments for the resistant line, TZAR101. PROC Mixed factorial analysis of variance with $\alpha = 0.05$ was used to determine contrasts of type 3 fixed effects for data interpretation. Error bars represent the standard error.

treatment. Expression in the wounded sample increased slightly from 1 to 6 DAI but did not show any variation at later time points. In TZAR101, there was no significant induction of expression at 1 and 4 DAI in response to inoculation or in the wounded treatment from 6 to 18 DAI. However, the level of expression for all treatments was lower at time points between 4 and 18 DAI in B73 in comparison to TZAR101, giving significance to the difference between the varietal responses ($p = 0.0181$). Treatments were found to result in significant changes in gene expression ($p < 0.0001$), and there was a significant two-way interaction of treatment x DAI ($p = <0.0001$). There was also a significant three-way interaction of variety x treatment x DAI ($p = 0.0012$) (Appendix A: Table A.1).

The expression levels of the gene annotated MZ00021479, identified as a *ZmWRKY53* isoform, were also analyzed over the time course in both the susceptible B73 line (Figure 4.3; Graph A) and the resistant TZAR101 line (Figure 4.3; Graph B). In B73, this *ZmWRKY53* gene was significantly induced by inoculation at 4 DAI and in conjunction with wounding at 10 DAI in comparison to the non-treated control. Expression levels were not significantly in the inoculated treatment compared to the non-treated control at 18 DAI. In TZAR101, there was an induction in expression at 2, 4, and 10 DAI in response to inoculation, beginning earlier than that in B73. No significant difference in expression levels between the treatments at the other time points was detected. Overall, the expression levels for all treatments in B73 and TZAR101 were similar, indicated by a marginally significant varietal effect in the statistical analysis ($p = 0.0648$). There was a significant treatment effect ($p = 0.0057$), two-way interaction of treatment x DAI ($p = 0.0361$), and three-way interaction of variety x treatment x DAI ($p = 0.0046$) (Appendix A: Table A.2).

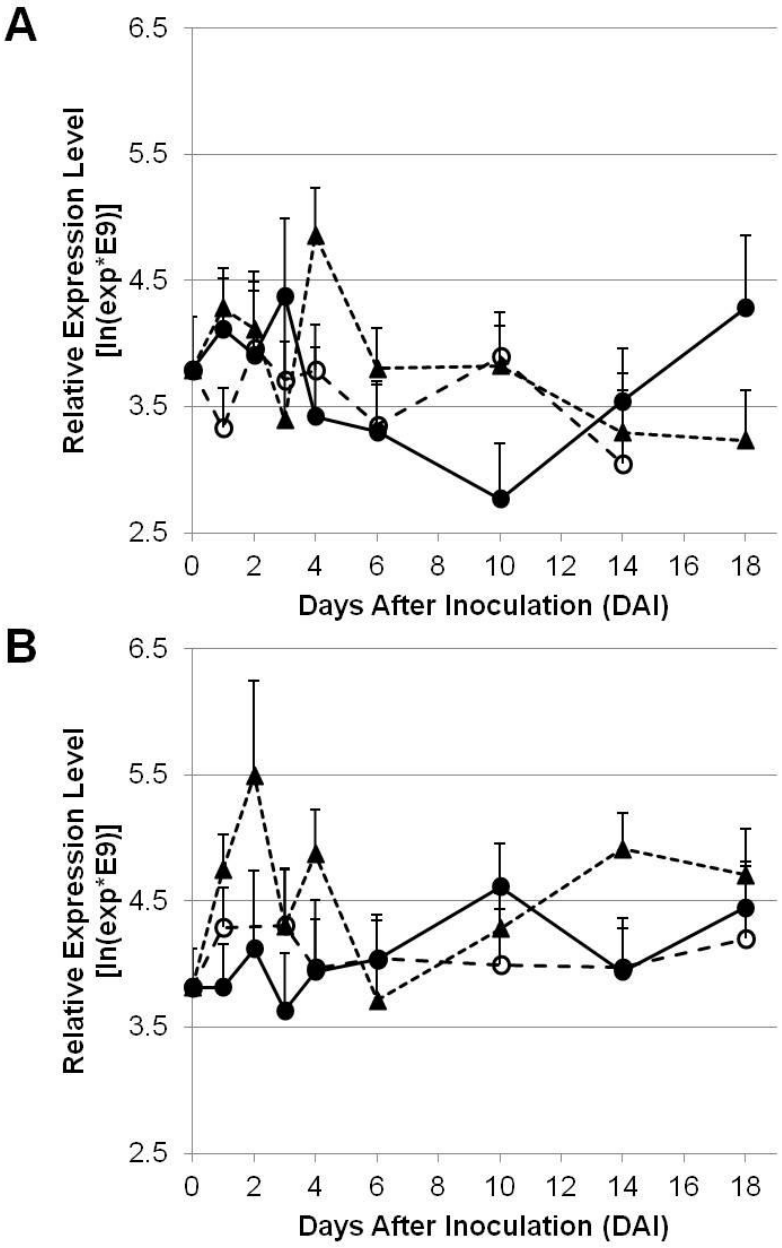

Figure 4.3 Relative expression levels of the *ZmWRKY53* gene (Microarray Accession: MZ00021479). Plotted values were calculated by taking the natural logarithm of the product of the relative expression of the *ZmWRKY53* gene by $1.0x10^9$ over the time course. Data points represent the average of the relative expression levels for each treatment and time point for all available years and plots. A. Relative expression levels over the time course in non-treated controls (●), wounded (○), and inoculated (▲) treatments for the susceptible line, B73. B. Relative expression levels over the time course in non-treated controls (●), wounded (○), and inoculated (▲) treatments for the resistant line, TZAR101. PROC Mixed factorial analysis of variance with $\alpha = 0.05$ was used to determine contrasts of type 3 fixed effects for data interpretation. Error bars represent the standard error.

Expression levels of the gene annotated MZ00026377, identified as the *ZmWRKY21* gene, were also compared between the susceptible B73 line (Figure 4.4; Graph A) and the resistant TZAR101 line (Figure 4.4; Graph B) over the time course of *A. flavus* inoculation. In B73, the *ZmWRKY21* expression was significantly reduced in response to wounding and inoculation at 3 DAI compared to the non-treated control. However, this is likely due to the lack of enough replications since the gene expression data at 3 DAI was from only one year of sampling (2011) and high year to year variation may be present. In TZAR101, there was a slight suppression in the wounded and inoculated treatment expression levels at 3 DAI compared to the non-treated control, but not significantly. For all remaining time points in both B73 and TZAR101, there were no significant differences in expression levels in response to any treatment. This resulted in no significant varietal effect in the statistical analysis ($p = 0.5153$). There were significant effects caused by treatment ($p = 0.0057$) and DAI ($p = 0005$). The two-way interactions between variety x treatment ($p = 0.0733$) and variety x DAI ($p = 0.0681$) were marginally significant, while that of treatment x DAI was significant ($p = 0.0071$). The three-way interaction between variety, treatment, and DAI was also significant ($p = 0.0159$) (Appendix A: Table A.3).

Expression levels of *ZmWRKY68* gene (MZ00042391) over the time course were analyzed in both the susceptible B73 line (Figure 4.5; Graph A) and the resistant TZAR101 line (Figure 4.5; Graph B). The *ZmWRKY68* gene displayed an expression pattern similar to that of *ZmWRKY21* in the non-treated control. It showed elevated expression levels at 0 and 3 DAI in B73. However, there were some differences in that there was a slight, induction in response to inoculation at 4 DAI, though marginally significant, in B73. In TZAR101, there was a significant suppression of the *ZmWRKY68* gene in responding to wounding, and *A. flavus* inoculation

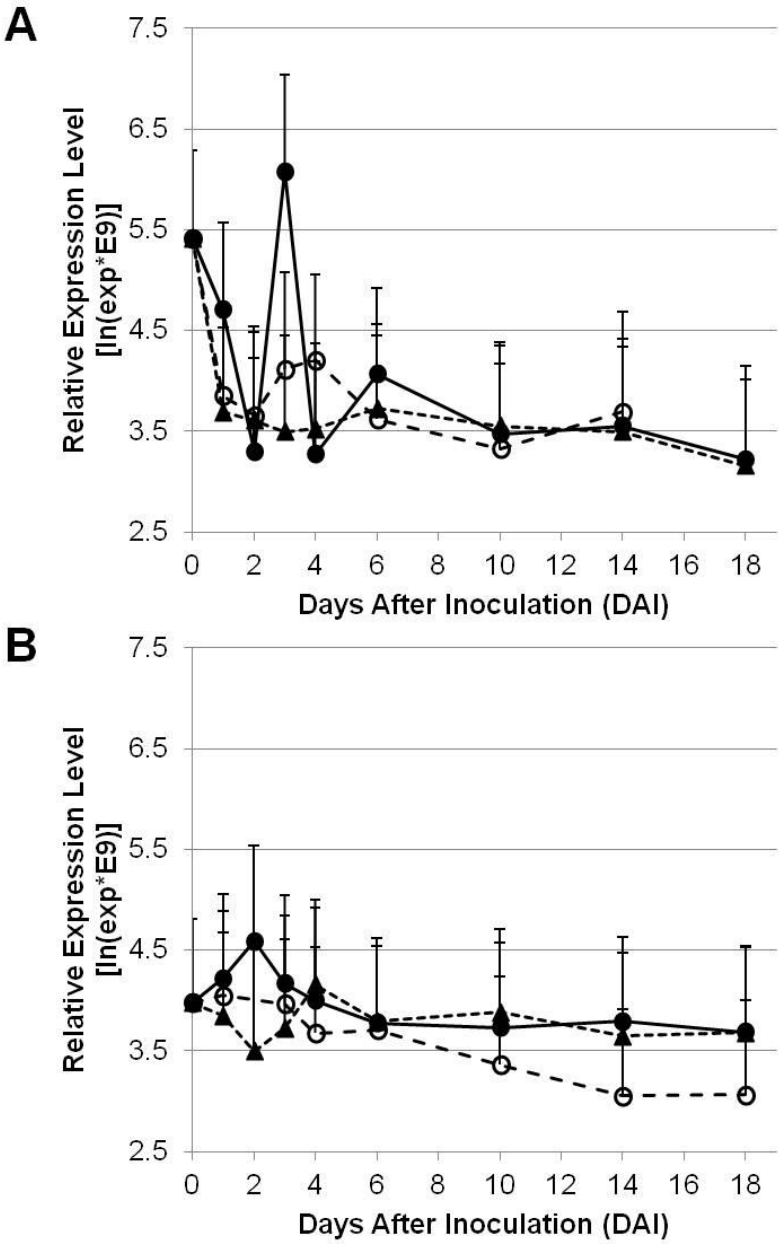

Figure 4.4 Relative expression levels of the *ZmWRKY21* gene (Microarray Accession: MZ00026377). Plotted values were calculated by taking the natural logarithm of the product of the relative expression of the *ZmWRKY21* gene by $1.0x10^9$ over the time course. Data points represent the average of the relative expression levels for each treatment and time point for all available years and plots. A. Relative expression levels over the time course in non-treated controls (●), wounded (○), and inoculated (▲) treatments for the susceptible line, B73. B. Relative expression levels over the time course in non-treated controls (●), wounded (○), and inoculated (▲) treatments for the resistant line, TZAR101. PROC Mixed factorial analysis of variance with $\alpha = 0.05$ was used to determine contrasts of type 3 fixed effects for data interpretation. Error bars represent the standard error.

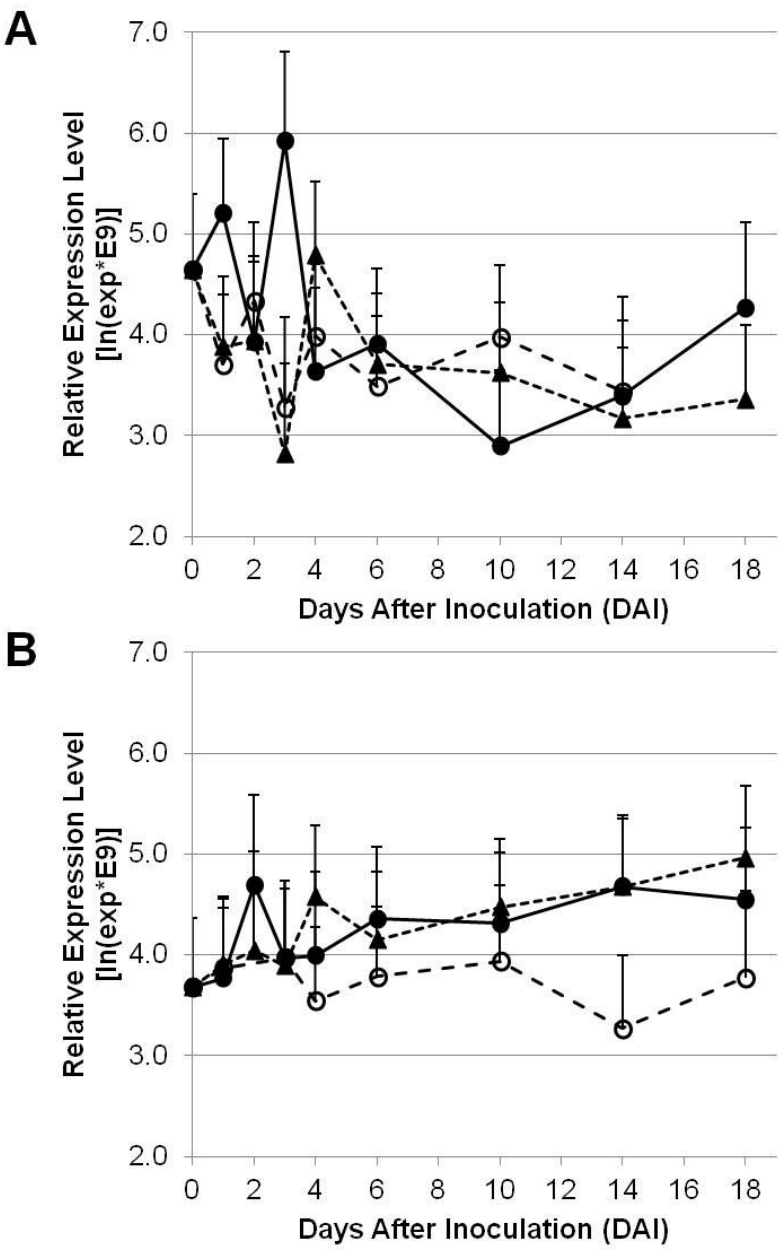

Figure 4.5 Relative expression levels of the *ZmWRKY68* gene (Microarray Accession: MZ00042391).Plotted values were calculated by taking the natural logarithm of the product of the relative expression of the *ZmWRKY68* gene by $1.0x10^9$ over the time course. Data points represent the average of the relative expression levels for each treatment and time point for all available years and plots. A. Relative expression levels over the time course in non-treated controls (●), wounded (○), and inoculated (▲) treatments for the susceptible line, B73. B. Relative expression levels over the time course in non-treated controls (●), wounded (○), and inoculated (▲) treatments for the resistant line, TZAR101. PROC Mixed factorial analysis of variance with $\alpha = 0.05$ was used to determine contrasts of type 3 fixed effects for data interpretation. Error bars represent the standard error.

appeared to increase its expression level back to that in the control at 14 DAI, though only marginally significant. There was no significant difference between the varieties ($p = 0.7105$) and no significant effect of DAI on expression levels ($p = 0.8587$), likely due to high variation between time points. The application of the treatments had a significant effect on expression ($p = 0.0084$), and all two- and three-way interactions between variety, treatment, and DAI were significant ($p < 0.009$) except for the two-way interaction between variety x DAI which was marginally significant ($p = 0.0766$) (Appendix A: Table A.4).

Expression levels of the second *ZmWRKY53* gene (MZ00042508) over the time course were also analyzed in both the susceptible B73 line (Figure 4.6; Graph A) and the resistant TZAR101 line (Figure 4.6; Graph B). This isoform of *ZmWRKY53* displayed a different pattern of expression than that of MZ00021479. This *ZmWRKY53* gene, hence designated *ZmWRKY53.1*, displayed a slight induction in response to inoculation at 2 and 4 DAI in both B73 and TZAR101, marginally significant only at 4 DAI in B73. No significant variation in expression among the treatments was observed for B73 at the other time points. In TZAR101, expression was induced to a similar degree in response to both wounding and inoculation and significantly at 14 DAI above the non-treated control, suggesting the induction was due to wounding rather than *A. flavus* inoculation. Overall, the expression levels of *ZmWRKY53.1* in B73 were significantly higher than in TZAR101 for all treatments and time points ($p < 0.0001$). Treatments were shown to have a significant effect on expression levels ($p = 0.0165$), and there was a significant interaction between treatment x DAI ($p = 0.0002$). All other two- and three-way interactions were insignificant (Appendix A: Table A.5).

Finally, the expression levels of the *ZmWRKY67* gene (PTZm631 from Han et al. 2010) were analyzed over the time course in both the susceptible B73 line (Figure 4.7; Graph A) and

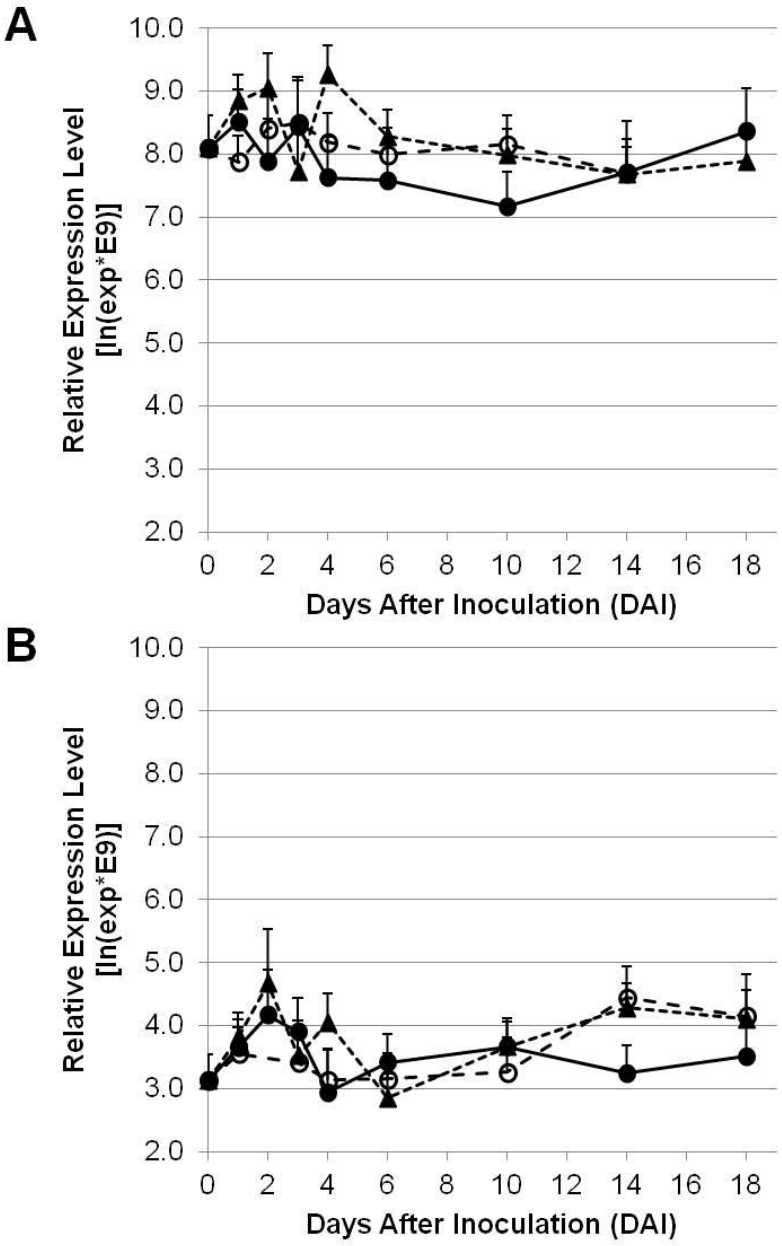

Figure 4.6 Relative expression levels of the *ZmWRKY53.1* gene (Microarray Accession: MZ00042508). Plotted values were calculated by taking the natural logarithm of the product of the relative expression of the *ZmWRKY53.1* gene by $1.0x10^9$ over the time course. Data points represent the average of the relative expression levels for each treatment and time point for all available years and plots. A. Relative expression levels over the time course in non-treated controls (●), wounded (○), and inoculated (▲) treatments for the susceptible line, B73. B. Relative expression levels over the time course in non-treated controls (●), wounded (○), and inoculated (▲) treatments for the resistant line, TZAR101. PROC Mixed factorial analysis of variance with $\alpha = 0.05$ was used to determine contrasts of type 3 fixed effects for data interpretation. Error bars represent the standard error.

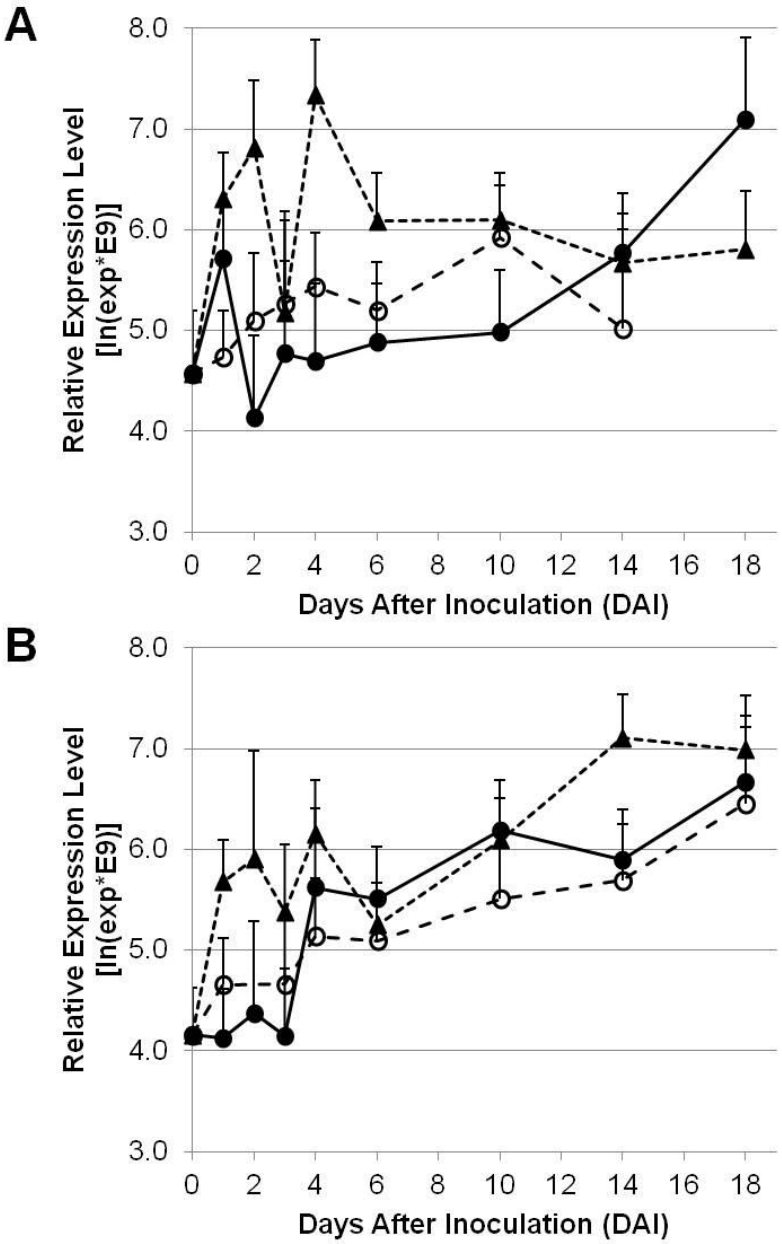

Figure 4.7 Relative expression levels of the *ZmWRKY67* gene (Accession: PTZm631, from Han et al. 2010). Plotted values were calculated by taking the natural logarithm of the product of the relative expression of the *ZmWRKY67* gene by $1.0x10^9$ over the time course. Data points represent the average of the relative expression levels for each treatment and time point for all available years and plots. A. Relative expression levels over the time course in non-treated controls (●), wounded (○), and inoculated (▲) treatments for the susceptible line, B73. B. Relative expression levels over the time course in non-treated controls (●), wounded (○), and inoculated (▲) treatments for the resistant line, TZAR101. PROC Mixed factorial analysis of variance with $\alpha = 0.05$ was used to determine contrasts of type 3 fixed effects for data interpretation. Error bars represent the standard error.

the resistant TZAR101 line (Figure 4.7; Graph B). The *ZmWRKY67* gene showed early induction from 0 to 6 DAI in both B73 and TZAR101. The initial induction in expression in response to inoculation was greater in B73 compared to TZAR101. Expression of the *ZmWRKY67* gene in the non-treated control exhibited a gradual developmental increase over the time course in both B73 and TZAR101; however, this occurred more rapidly in TZAR101 with a sharp increase at 4 DAI. Expression levels in the inoculated treatment returned to initial levels and remained constant following an early induction in B73, whereas the expression in TZAR101 returned to initial levels then increased to a higher level later in the time course from 10 to 18 DAI. This increase was comparable to that of the early induction observed in B73 and was significantly greater than that of the non-treated control and wounded treatments in TZAR101 at 14 DAI. Overall, there was no significant difference between the two varieties ($p = 0.8995$), or in the two-way interactions between variety x treatment ($p = 0.8426$) and variety x DAI ($p = 0.7451$). However, the two-way interaction between treatment x DAI ($p = 0.0048$), the three-way interaction between variety x treatment x DAI ($p = 0.0225$), the application of the treatments ($p < 0.0001$), and DAI ($p = 0.0033$) were all significant (Appendix A: Table A.6).

4.4 Expression Analysis of Prospective Pathway Genes

In addition to the maize WRKY TF genes, the expression levels of several defense pathway genes were also examined. The expression levels of the *ZmPR-1* gene in both the susceptible B73 line (Figure 4.8; Graph A) and the resistant TZAR101 line (Figure 4.8 Graph B) were analyzed over the time course of *A. flavus* inoculation. The *ZmPR-1* gene displayed a significant similar suppression in expression at early time points in the wounded and inoculated treatments compared to the non-treated control from 0 to 4 DAI in B73, suggesting the observed suppression in *ZmPR-1* expression was due to wounding rather than *A. flavus* inoculation.

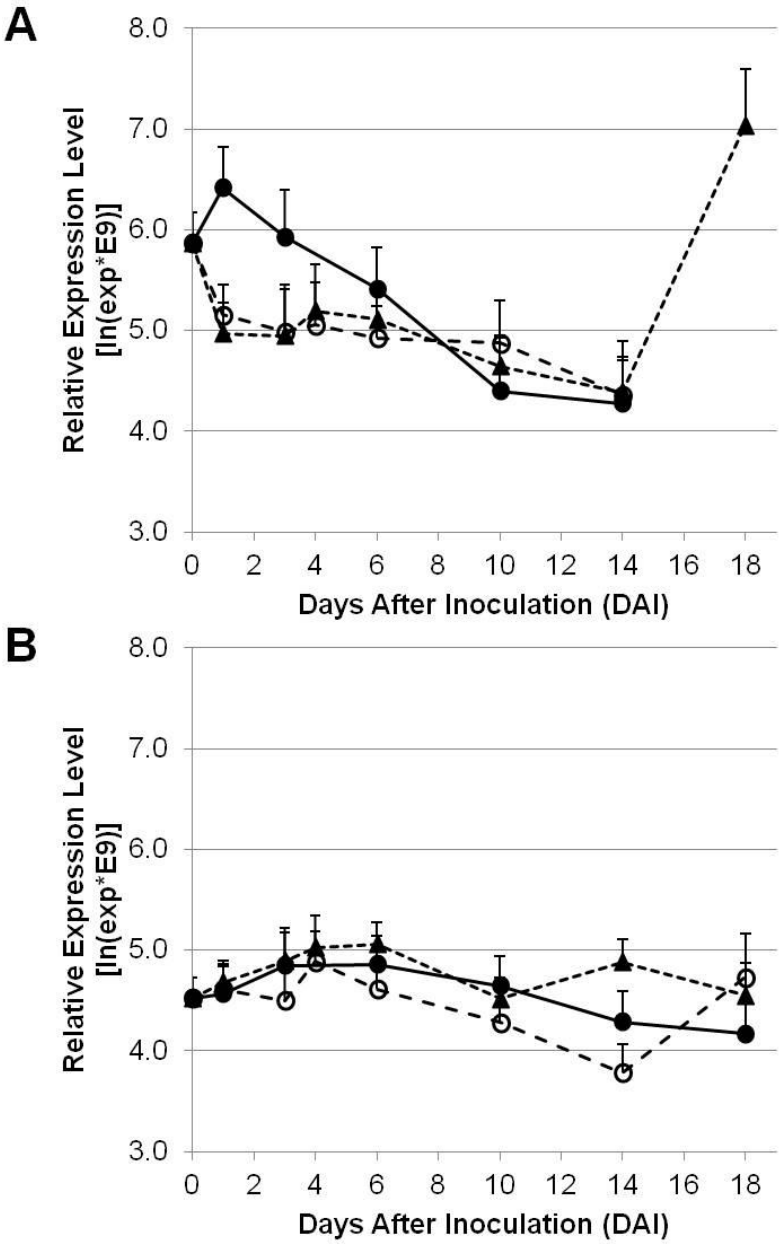

Figure 4.8 Relative expression levels of the *ZmPR-1* gene. Plotted values were calculated by taking the natural logarithm of the product of the relative expression of the *ZmPR-1* gene by $1.0x10^9$ over the time course. Data points represent the average of the relative expression levels for each treatment and time point for all available years and plots. A. Relative expression levels over the time course in non-treated controls (●), wounded (○), and inoculated (▲) treatments for the susceptible line, B73. B. Relative expression levels over the time course in non-treated controls (●), wounded (○), and inoculated (▲) treatments for the resistant line, TZAR101. PROC Mixed factorial analysis of variance with $\alpha = 0.05$ was used to determine contrasts of type 3 fixed effects for data interpretation. Error bars represent the standard error.

ZmPR-1 expression in the wounded and inoculated treatments was not significantly different from the non-treated control in B73 from 6 to 14 DAI. At 18 DAI, there was an increase in expression in the inoculated samples; however, control samples were not available for comparison at this time point. In contrast, *ZmPR-1* expression levels in TZAR101 were not significantly induced or suppressed in response to any treatment at all time points except at 14 DAI where there was a marginally significant increase in expression in inoculated samples compared to wounding or the non-treated control. The contrasting patterns of *ZmPR-1* expression between B73 and TZAR101 indicated a significant difference in the responses of the two varieties to wounding ($p = 0.0155$). Treatment application ($p = 0.0225$) as well as the time course ($p = 0.0263$) had significant effects on expression levels. In addition, the two-way interactions between variety x DAI ($p = 0.0160$) and treatment x DAI ($p = 0.0029$) were significant, while the two-way interaction between variety x treatment ($p = 0.3315$) and the three-way interaction between variety x treatment x DAI ($p = 0.3306$) were not significant (Appendix A: Table A.7).

Expression levels of the *ZmNPR1* gene were also analyzed in this study (Figure 4.9). The expression levels of *ZmNPR1* mimicked that of *Zm*PR-1 for the non-treated control and inoculated treatments at all time points in B73 (Figure 4.9; Graph A), showing significant suppression in gene expression in the wounded and inoculated treatments compared to the non-treated control from 0 to 3 DAI. The inoculated treatment did not cause any significant variation in *ZmNPR1* expression compared to the wounding treatment in B73 until 18 DAI where there was a similar increase in expression as observed for *ZmPR-1*. Again, no control samples were available for comparison at the 18 DAI time point for *ZmNPR1*. However, in TZAR101 (Figure 4.9; Graph B), *ZmNPR1* expression was induced at later time points by inoculation compared to

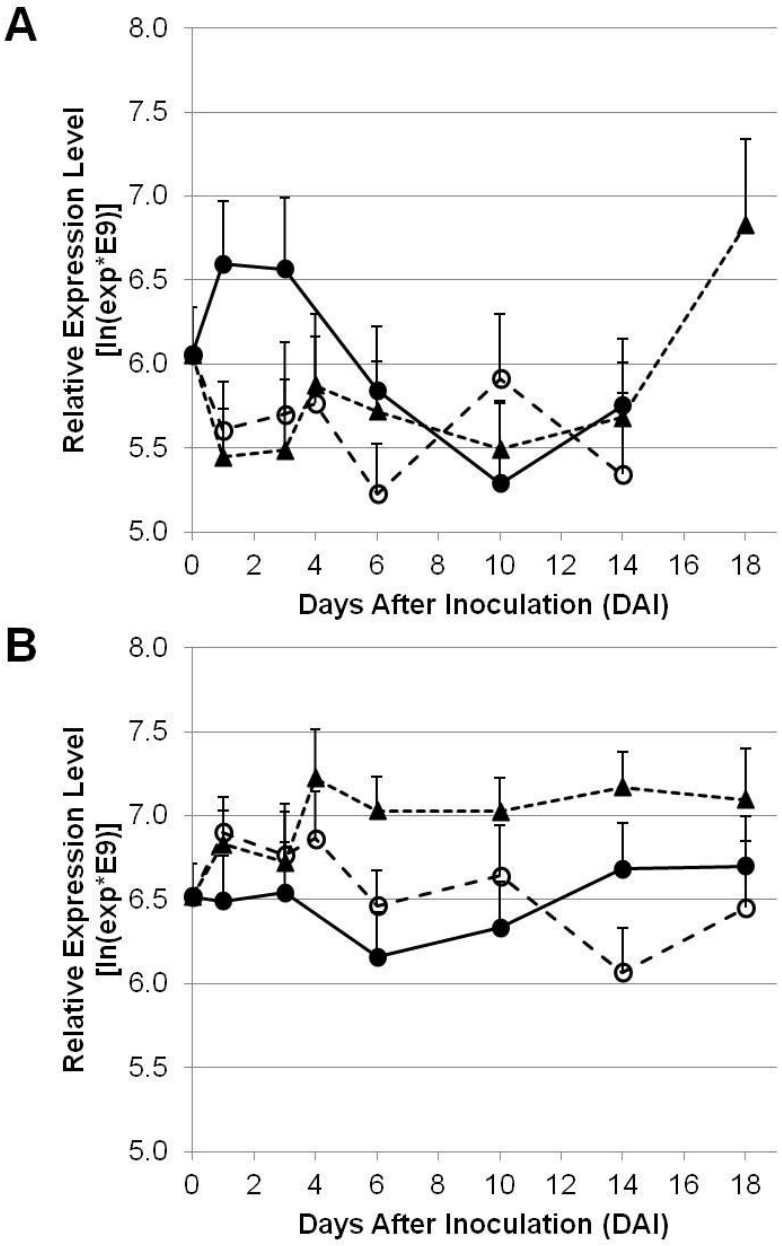

Figure 4.9 Relative expression levels of the *ZmNPR1* gene. Plotted values were calculated by taking the natural logarithm of the product of the relative expression of the *ZmNPR1* gene by $1.0x10^9$ over the time course. Data points represent the average of the relative expression levels for each treatment and time point for all available years and plots. A. Relative expression levels over the time course in non-treated controls (●), wounded (○), and inoculated (▲) treatments for the susceptible line, B73. B. Relative expression levels over the time course in non-treated controls (●), wounded (○), and inoculated (▲) treatments for the resistant line, TZAR101. PROC Mixed factorial analysis of variance with $\alpha = 0.05$ was used to determine contrasts of type 3 fixed effects for data interpretation. Error bars represent the standard error.

wounding or non-treated controls, a pattern which is different from that observed for *ZmPR-1*. The expression of *ZmNPR1* in response to inoculation was significantly higher than the non-treated control in TZAR101 from 4 to 10 DAI and significantly higher than the wounded treatment beginning at 6 DAI. Expression of *ZmNPR1* was also suppressed at 14 and 18 DAI in the wounded treatment in comparison to the non-treated control.

Overall, the expression levels of *ZmNPR1* in the non-treated control and inoculated treatments remained elevated in TZAR101 over the duration of the time course, particularly later than 3 DAI, in comparison to those observed in B73, resulting in a significant effect of variety on expression levels ($p = 0.0051$). Treatment application was shown to have a significant effect on expression levels ($p = 0.0264$), while the time course had no significant effect on expression levels ($p = 0.2324$). In addition, the two-way interactions between variety x treatment ($p = 0.0043$) and treatment x DAI ($p = 0.0414$) were significant. The remaining two-way interaction, variety x DAI ($p = 0.5056$), and the three-way interaction between variety x treatment x DAI ($p = 0.1569$) were not significant (Appendix A: Table A.8).

Finally, the expression levels of the *ZmERF1* gene, a marker gene representing the JA and ethylene signal transduction pathways (Lorenzo et al. 2003), were analyzed over the time course in both the susceptible B73 line (Figure 4.10; Graph A) and the resistant TZAR101 line (Figure 4.10; Graph B). The *ZmERF1* gene displayed a decreasing trend in expression levels developmentally in the non-treated controls from 0 to 10 DAI in B73 followed by an increase in expression at 14 DAI to initial levels. Neither wounding nor inoculation by *A. flavus* significantly affected its expression, except at 3 DAI when suppression of *ZmERF1* expression by inoculation was observed. *ZmERF1* expression levels in TZAR101, in contrast, remained consistent for the duration of the time course with a slight elevation in expression in response to

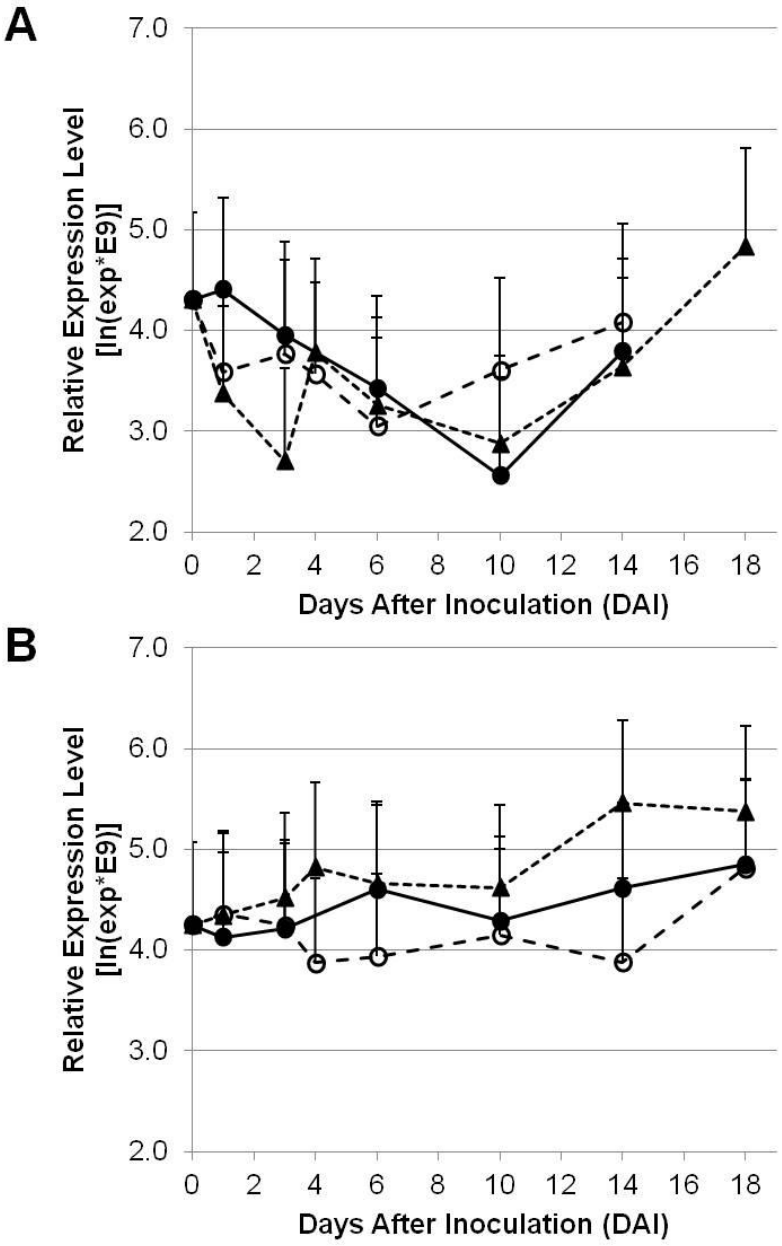

Figure 4.10 Relative expression levels of the *ZmERF1* gene. Plotted values were calculated by taking the natural logarithm of the product of the relative expression of the *ZmERF1* gene by $1.0x10^9$ over the time course. Data points represent the average of the relative expression levels for each treatment and time point for all available years and plots. A. Relative expression levels over the time course in non-treated controls (●), wounded (○), and inoculated (▲) treatments for the susceptible line, B73. B. Relative expression levels over the time course in non-treated controls (●), wounded (○), and inoculated (▲) treatments for the resistant line, TZAR101. PROC Mixed factorial analysis of variance with $\alpha = 0.05$ was used to determine contrasts of type 3 fixed effects for data interpretation. Error bars represent the standard error.

inoculation at 14 DAI compared to the wounding. Despite the observed different patterns of *ZmERF1* expression between the two varieties, no significant difference was detected between the two varieties ($p = 0.1522$). This is likely due to the high degree of variability observed between time points as indicated by the standard error. The time course did have a significant effect on expression levels ($p = 0.0032$), while treatment application did not ($p = 0.1067$). In addition, the two-way interactions between variety x treatment ($p = 0.0017$) and variety x DAI ($p = 0.0410$) were significant, while the remaining two-way interaction, treatment x DAI ($p = 0.1034$), was not significant. The three-way interaction between variety x treatment x DAI ($p = 0.0729$) was found to be marginally significant (Appendix A: Table A.9).

5. DISCUSSION

5.1 Potential Functions of WRKY Transcription Factor Genes

Resistance of maize to colonization by *Aspergillus flavus* is a quantitative trait expressed by the combined effects of multiple defense mechanisms mediated through various signaling pathways (Brooks et al. 2005; Kelley et al. 2012; Paul et al. 2003). This has been shown through the examination of both the proteome and expression profiles of resistant and susceptible maize varieties, along with studies showing the environmental influences on colonization and subsequent aflatoxin production (Chen et al. 2012a; Kelly et al. 2012; Luo et al. 2008; Scully et al 2009). Several studies have uncovered the function of several proteins such as β-1,3-glucanases, chitinases, glyoxalase I (GLX-I), *Zm*PR10, *Zm*PR10.1, ribosome inactivating proteins, and zeamatin which play partial roles in the overall resistance phenotype (Chen et al. 2004, 2006, 2010; Huynh et al. 1992; Guo et al. 1997; Lozovaya et al. 1998; Mauch et al. 1988; Walsh et al. 1991; Xie et al. 2010). It has also been found that drought and heat stress can either enhance or diminish the resistance of some maize varieties to *A. flavus* infection indicating the potential involvement of abiotic stress response pathways in regulating resistance responses (Kebede et al. 2012). In addition, recent transcriptional profiling studies have shown that components of signaling pathways mediated by several phytohormones including abscisic acid (ABA), ethylene, jasmonic acid (JA), and salicylic acid (SA) are also regulated in maize in response to *A. flavus* infection (Kelley et al. 2012; Luo et al. 2010, 2011).

Luo et al. (2011) examined the expression profiles of resistant and susceptible maize kernels and showed that the expressions of several *Zm*WRKY transcription factors were significantly regulated in response to *A. flavus* infection. In recent studies, WRKY transcription factors have been found to be involved in defense regulation in model plant species against

several different classes of plant pathogens including hemibiotrophs, such *as Pseudomonas syringae* pv. *tomato* (Higashi et al. 2008), which alternate from biotrophic lifestyles early in infection to necrotrophic lifestyles later (Glazebrook, 2005), and necrotrophs, such as *Botrytis cinerea* (Birkenbihl et al. 2012), which kill host tissues to derive nutrients (Glazebrook, 2005).WRKY transcription factors have also been found to be involved in regulating responses to abiotic stress including cold stress, drought stress, heat stress, herbivore feeding, oxidative stress, and wounding (Chen et al. 2012b).

In this study, we examined the expression of several of identified *Zm*WRKY transcription factors in both resistant (TZAR101) and susceptible (B73) maize varieties in response to *A. flavus* inoculation under field conditions. We also examined the expression of the maize orthologs of several *A. thaliana* genes known to be associated with some hormone signaling pathways, *AtPR-1* and *AtNPR1* which are associated with SA-mediated defense pathways (Spoel et al 2007; Zhang et al. 1999) and *AtERF1* which serves as the intersection of JA- and ethylene-mediated defense pathways (Lorenzo et al. 2003), to determine which signaling pathways are regulated in response to *A. flavus* inoculation.

The expression levels of the selected WRKY transcription factors and pathway indicator genes were analyzed in both varieties over the time course of the experiment. In the resistant variety, TZAR101, the putative *ZmWRKY19* (AFW81188.1), *ZmWRKY67*, *ZmWRKY68*, *ZmNPR1*, and *ZmERF1* were expressed at higher levels, both constitutively and upon inoculation than in the susceptible line, B73, particularly after 4 to 6 DAI. The remaining genes showed significant differences in expression in response to treatments across the time course in both TZAR101 and B73 with the exception of *ZmWRKY21* (Figure 4.4; MZ00019797). This gene showed no significant regulation in response to the treatments in the resistant line except at 3

DAI. Some variation was observed at 3 DAI in several of the other genes examined in this study, most likely due to lack of sufficient replications since these data for 3 DAI were from a single year, 2011.

However, variation in *ZmWRKY21* expression as the result of environmental effects can't be ruled out. The homolog of *Zm*WRKY21 in *A. thaliana*, as determined by a BLASTp search, *At*WRKY39 (NP_566236.1), functions in regulating thermotolerance (Li et al. 2010). *At*WRKY39 has been demonstrated to interact with calmodulin, to be induced by H_2O_2 accumulation, and potentially be regulated by ethylene-dependent signaling pathways (Hass et al. 2004; Li et al. 2010; Park et al. 2005; Popescu et al. 2007; Vanderauwera et al. 2005). The expression of *AtWRKY39* was also found not to be induced in response to inoculation by virulent strains of *Pseudomonas syringae* (Journot-Catalino et al. 2006). This, combined with the lack of significant induction observed in response to *A. flavus* inoculation may indicate that *Zm*WRKY21 may respond primarily to environmental stress and not to pathogen infections.

*Zm*WRKY19 showed sequence homology to *At*WRKY53 (NCBI: NP_194112.1; TAIR: AT4G23810.1; BLASTp: Coverage = 43%, ID = 47%; BLASTx: Coverage = 14%, ID = 80%) in the BLAST search. *At*WRKY53 has been shown to function in response to oxidative stress and its expression can be regulated either through the action of NPR1/TGA transcription factor complexes or through MAPK pathway components, such as MEKK1 (Eulgem and Somssich, 2007; Rushton et al. 2010; Miao et al. 2007). The expression of *AtWRKY53* and *AtWRKY33* are also both regulated by MPK3 and MPK6 in response to chitin perception (Wan et al. 2004), and *At*WRKY53 has been shown to interact with calmodulin (Popescu et al. 2007). *At*WRKY53 can also bind to the promoters of genes encoding antioxidant enzymes including several isoforms of catalase (Miao et al. 2004). In B73, the *Zm*WRKY19 gene (Figure 4.2; Graph A) was induced by

wounding but to a greater extent by *A. flavus* inoculation from 0 to 6 DAI. An induction in expression levels in response to wounding and inoculation was also observed from 6 to 14 DAI, though to a lesser degree than that observed from 0 to 6 DAI. In contrast, in TZAR101 (Figure 4.2; Graph B), the gene was induced to levels comparable to that of B73 by inoculation; however, no decrease in expression was observed at later time points. In addition, the expression of *ZmWRKY19* was higher during normal developmental conditions in TZAR101 than in B73 over the time course.

If *Zm*WRKY19 functions in a similar manner as *At*WRKY53, it is possible that resistant varieties of maize similar to TZAR101 may accumulate antioxidant enzymes to a greater extent than susceptible lines like B73. Evidence supporting this speculation has been observed in several recent studies. Magbanua et al. (2007) found that resistant maize varieties Mp313E and Mp420 possessed greater catalase activity and accumulated lower levels of H_2O_2 in response to *A. flavus* infection compared to the intermediate variety Tx601 or the susceptible varieties Mp339, SC212m, or SC229. They also found that the resistant varieties also accumulated higher levels of SA than the susceptible lines, although infection by *A. flavus* tended to reduce the accumulation of SA. In two additional studies, Pechanova et al. (2011) and Chen et al. (2012a), isoforms of superoxide dismutase have been found to be induced in resistant varieties. Chen et al. (2007) also found that a homolog to the barley 1-Cys peroxiredoxin antioxidant (PER1) protein was expressed at higher levels in resistant maize varieties. With this, the increase in expression of the *AtWRKY53* homolog in maize, *ZmWRKY19*, may be regulating the expression of genes encoding such antioxidant enzymes and be caused by a similar regulatory response as *At*WRKY53 and *At*WRKY33 through MPK3 and MPK6 in response to chitin (Wan et al. 2004). The accumulation of these antioxidant proteins may confer resistance to abiotic stresses that

cause negative effects through the production of ROS, such as drought or heat stress (Hu et al. 2008, 2010; Volkov et al. 2006), which correlates to enhanced *A. flavus* resistance (Kebede et al. 2012; Pechanova et al. 2011).

In maize, both *ZmWRKY53* and *ZmWRKY53.1* displayed significant induction in response to *A. flavus* inoculation from 0 to 6 DAI. However, *ZmWRKY53.1* (Figure 4.6) was expressed at significantly higher levels regardless of treatment in B73 (Figure 4.6; Graph A) than in TZAR101 (Figure 4.6; Graph B). For *ZmWRKY53* (Figure 4.3), the overall expression levels were similar between the two varieties. However, there were variations in the expression patterns between the two varieties. In TZAR101 (Figure 4.3; Graph B), there was an earlier, more pronounced induction in *ZmWRKY53* expression at 2 DAI continuing to 4 DAI, earlier than that observed in B73 (Figure 4.3; Graph A). Also, the constitutive level of *ZmWRKY53* expression in the TZAR101 non-treated control was higher than that of B73 at 10 DAI. When taken together, the overall patterns within the individual varietal responses for both genes are similar with the exception of later induction of *ZmWRKY53.1* in response to wounding in TZAR101. This may indicate that these two genes may possess similar promoters. The reason, however, for the elevated level of expression of *ZmWRKY53.1* in B73 may simply be the presence of multiple functional copies of the gene in the genome of B73. Based on sequence homology search of maize genome, at least two regions showing partial homology to this gene may be present in B73. The number of copies may be fewer in TZAR101; however, a complete sequencing of the genome of TZAR101 will be necessary to confirm this hypothesis (See Appendix C).

The two WRKY homologs named *ZmWRKY53* (MZ00021479) and *ZmWRKY53.1* (MZ00042508), which showed differential expression in an earlier microarray study (Luo at el. 2011), both exhibited similar levels of homology to *At*WRKY33 (AAM34736.1; BLASTx: 44%

and 45% identity, respectively) and *Os*WRKY53 (NP_001055252.1; 68% and 69% identity, respectively) in a BLASTp search. In order to determine which *Zm*WRKY53 isoform is functionally similar to *At*WRKY33 and *Os*WRKY53, the amino acid sequences from the Genebank for each isoform were searched using BLASTp in the Plant Transcription Factor Database (Perez-Rodriguez et al. 2010) resulting in the identification of accessions GRMZM2G012724_P01 corresponding to *Zm*WRKY53 and GRMZM2G150441_P03 corresponding to *Zm*WRKY53.1. These were then compared to the findings of a recent study by Wei et al. (2012) who determined the functional orthologs of maize WRKY transcription factors based on homology analysis and gene structure. Their findings indicate that the accession GRMZM2G012724_P01, which they designated as *Zm*WRKY70.1 and is referred to as *Zm*WRKY53 in this study, is the putative functional ortholog of *At*WRKY33 and *Os*WRKY53. These findings combined with the dramatic difference in the varietal expression patterns of *Zm*WRKY53.1 compared to *Zm*WRKY53 observed in this study, and the relatively low level of homology between the putative amino acid sequences of these proteins (83%) may indicate that *Zm*WRKY53.1 possesses a different function than *Zm*WRKY53 and is in need of further characterization.

*At*WRKY33 is a key regulator in promoting the expression of JA-based defenses in response to necrotrophic pathogen infections while suppressing the expression of SA-based defenses, such as PR proteins, which classically counter biotrophic pathogens (Birkenbihl and Somssich, 2011; Birkenbihl et al.2012; Chujo et al. 2007; Kishi-Kaboshi et al. 2010; Yu et al. 2010). Conversely, the overexpression of *OsWRKY53* has been shown to enhance the expression of PR genes such as *OsPR-5* and *OsPR-14* as well as other defense genes including chitinase and peroxidase encoding genes (Chujo et al. 2007; van Eck, 2011). *AtWRKY33* is regulated by a

MAP Kinase pathway responding to PAMP-triggered immune signals through those downstream of MEKK1,MPK4 and MKS1, which function to negatively regulate the expression of *At*WRKY33-regulated genes by complexing with the *At*WRKY33 protein (Andreasson et al. 2005; Eulgem and Somssich, 2007; Qiu et al. 2008; Rushton et al. 2010). Additional MAP Kinases, MPK3 and MPK6, also directly interact with *At*WRKY33 and activate it through phosphorylation in response to pathogen infection (Mao et al. 2011). A similar regulatory pathway involving MPK3 and MPK6 as found in *A. thaliana* for the regulation of phytoalexin biosynthesis has also been reported in rice (Kishi-Kaboshi et al. 2010). In addition, MPK3 and MPK6 have been implicated in the production of ROS in hypersensitivity induced cell death (Liu et al. 2007; Mao et al. 2011), and are responsive to chitin percepton independent of SA, JA, or ethylene signalling pathways (Wan et al. 2004; Zhang et al. 2002).

Expression of a maize homolog of *At*WRKY33, *Zm*WRKY53, may, given the described role of *At*WRKY33 (Birkenbihl and Somssich, 2011), enhance the expression of JA-based defenses. If true, elevated expression levels of JA-responsive defense-related genes would be expected with increased JA accumulation in the resistant variety compared to the susceptible variety. This was not observed by Magbanua et al. (2007) who saw no significant difference in JA content between immature kernels from varieties resistant and susceptible to *A. flavus* infection during kernel development at time points corresponding to the time course used in this study. This may indicate that the function of *Zm*WRKY53 may be similar to that of other orthologous proteins in other species such as the rice homolog, *Os*WRKY53 or the wheat homolog, *Ta*WRKY53 (AGF90798.1). A series of recent studies by van Eck (2011) characterized the function and regulation of *TaWRKY53* in response to aphid feeding and found that this gene was regulated by multiple proteins including a calmodulin-related calcium sensor

protein, an ultraviolet-B repressible protein, and a DUF584 protein, and propose the role of MAPK signalling in regulating the expression of *TaWRKY53* as seen in *AtWRKY33* and *OsWRKY53* due to homology between these orthologs (Kishi-Kaboshi et al. 2010; Mao et al. 2011; van Eck, 2011). van Eck (2011) also found that the *Ta*WRKY53 protein interacts with glutathione *S*-transferase (GST), which functions in conjunction with other antioxidant enzymes to counter stress-induced ROS (Gill and Tuteja, 2010), and was co-expressed along with a chitinase and a peroxidase, although they were unable to detect any interaction of *Ta*WRKY53 with the promoters of those genes.

Given these apparent functions of *Ta*WRKY53 and *Os*WRKY53, it is possible that *Zm*WRKY53 is regulated in a similar fashion and may have similar downstream targets such as chitinase or peroxidase genes. This seems plausible given the amino acid sequence homology observed among these WRKY transcription factors (Figure 5.1) and since chitinase and peroxidase proteins have been found to accumulate in the kernels of resistant maize lines in response to *A. flavus* infection (Chen et al. 2007; Moore et al. 2004). *Zm*WRKY53 may also play a role in phytoalexin biosynthesis given its orthology to *At*WRKY33 which is involved in camalexin biosynthesis in *A. thaliana* and regulated by MPK3 and MPK6 in a similar fashion observed for phytoalexin production *Os*WRKY53 (Kishi-Kaboshi et al. 2010; Mao et al. 2011). In addition, van Eck et al. (2010) suggests a potential role for *Ta*WRKY53 in regulating the expression of phenylalanine ammonia-lyase, which is involved in phytoalexin biosynthesis (Dixon et al. 2002). *Zm*WRKY53 may also promote the production of phytoalexins in maize, which, in the case of zealexin A1, has been shown to inhibit the growth of *A. flavus* in culture (Huffaker et al. 2011). However, further studies will be required to validate this potential role.

Figure 5.1 Alignment of amino acid sequences of homologs of *Zm*WRKY53 from other species. The amino acid sequences of the homologs of *Zm*WRKY53 from *A. thaliana* (AAM34736.1), rice (DAA05118.1), and wheat (AGF90798.1) as well as the *Zm*WRKY53.1 isoform were aligned using Clustal Omega (EMBL-EBI; http://www.ebi.ac.uk/ Tools/msa/clustalo/). The highlighted region indicates the positions of the WRKY DNA binding domains in the sequences.

```
ZmWRKY53        MASSTGSLEHGGFTFTPPPFITSFTELLSGAAADMVGAAGADHQERSPRGLFHRGATRGG
ZmWRKY53.1      MASSTGSLEHGGFTFTPPPFITSFTELLSSAG-DMLG-AGADQERSSPRGLFHRGA----
OsWRKY53        MASSTGGLDH-GFTFTPPPFITSFTELLSGGGGDLLGAGG---EERSPRGFSRGGARVG-
TaWRKY53        MSSSTGSLDHAGFTFTPPPFITSFTELLSGSGA--------GDAERPPRGFNR-----GG
AtWRKY33        -------------------MDN------SRTRQNMNGSANWSQQ------SGRTSTSSLE
                                    : .      *                    :

ZmWRKY53        GVGVPKFKSAQPPSLPISPPPMSPSSYFSIPPGLSPAELLDSPVLLHSSSNFFASPTTGA
ZmWRKY53.1      -RGVPKFKSAQPPSLPISPPPMSPSSYFAIPPGLSPAELLDSPVLLHSSSNILASPTTGA
OsWRKY53        -GGVPKFKSAQPPSLPLSPPPVSPSSYFAIPPGLSPTELLDSPVLLS-SSHILASPTTGA
TaWRKY53        RAGAPKFKSAQPPSLPISS----PFSCFSVPAGLSPAELLDSPVLLN-YSHILASPTTGA
AtWRKY33        DLEIPKFRSFAPSSISISPSLVSPSTCFS------PSLFLDSPAFVSSSANVLASPTTGA
                    ***:*  * *: :*     * : *:      *: :****.::   :..:*******

ZmWRKY53        IPAQRFDWKHAADLIASQSQQDDSRAAVGSAFNDFSFHAPTMP-------------AQTT
ZmWRKY53.1      IPAQRFDWKKAADLIASQSQQDGDSRAAAGGFDDFSFHTATSNAVRAH--------TTTT
OsWRKY53        IPAQRYDWKASADLIASQQDDS---------RGDFSFHTNSDA---MA--------AQPA
TaWRKY53        IPAQRCDWQASADLNTFQQDEL--------GLSGFSFHAVKSNA-TVN--------AQAN
AtWRKY33        LITNVTNQKGINEG--------DKSNNNNFNLFDFSFHTQSSGVSAPTTTTTTTTTTTT
                : ::  : :   :                    ****: .              :

ZmWRKY53        SFPSFKEQQQQQVEAAT----KSAVPSSNKASGGG-GGTKLEDGYNWRKYGQKQVKGSEN
ZmWRKY53.1      SLPSFEEEQQQQVEK-------AAVPSSNRASGGGNGNTKLEDGYNWRKYGQKQVKGSEN
OsWRKY53        SFPSFKEQEQQVVESSK----NGAAAASSNKS-GGGGNNKLEDGYNWRKYGQKQVKGSEN
TaWRKY53        CLPLFKEQQEQQQEEV--------VQVSNKSSSSSGNNKQVVDGYNWRKYGQKQVKGSEN
AtWRKY33        NSSIFQSQEQQKKNQSEQWSQTETRPNNQAVSYNGREQRKGEDGYNWRKYGQKQVKGSEN
                    *:.:::*  :             ..  *  .    :  ******************

ZmWRKY53        PRSYYKCTYHSCSMKKKVERSLADGRVTQIVYKGAHNHPKPLSTRRNSSGGVAAAEEQAA
ZmWRKY53.1      PRSYYKCTYHSCSMKKKVERALADGRITQIVYKGAHNHPKPLSTRRNSSGGGAAEELQAG
OsWRKY53        PRSYYKCTYNGCSMKKKVERSLADGRITQIVYKGAHNHPKPLSTRRNASSCATAAACAD-
TaWRKY53        PRSYYKCTYNNCSMKKKVERSLADGRITQIVYKGAHDHPKPLSTRRNSSGCAAVVAEDHT
AtWRKY33        PRSYYKCTFPNCPTKKKVERSL-EGQITEIVYKGSHNHPKPQSTRRSSSSSSTFHSAVY-
                ********:  *  ******:* :*::*:*****:*:**** ****.:*.  :

ZmWRKY53        NN----SSLSGCGG----------PEHSGG-ATAENSSVTFGDDEAENGSQ------RSGG
ZmWRKY53.1      NSSLSAVAAAGCTG----------PEHSG--ATAENSSVTFGDDEAENGSQ------RSDG
OsWRKY53        -----DLA-APGAG----------ADQYSA-ATPENSSVTFGDDEADNASH------RSEG
TaWRKY53        ------------NG----------SEH-SG-PTPENSSVTFGDDEAD--------------
AtWRKY33        NASLDHNRQASSDQPNSNNSFHQSDSFGMQQEDNTTSDSVGDDEFEQGSSIVSRDEEDCG
                                       :  .     :.:* :.**** :

ZmWRKY53        DEPDAKRWKAEDG---ENEGSSGAGGGKPVREPRLVVQTLSDIDILDDGFRWRKYGQKVV
ZmWRKY53.1      DEPDAKRWKQEDG---ENEGSSAGGGGKPVREPRLVVQTMSDIDILDDGFRWRKYGQKVV
OsWRKY53        DEPEAKRWKEDADNEGSSGGMGGGAGGKPVREPRLVVQTLSDIDILDDGFRWRKYGQKVV
TaWRKY53        -KPETKRRKEHGDNEGSSGGT--GGCGKPVREPRLVVQTLSDIDILDDGFRWRKYGQKVV
AtWRKY33        SEPEAKRWKGDNETNGG-----NGGGSKTVREPRIVVQTTSDIDILDDGYRWRKYGQKVV
                 :*::** * .            .. .* *****:**** *********:**********

ZmWRKY53        KGNPNPRSYYKCTTAGCPVRKHVERACHDARAVITTYEGKHNHDVPVGRGAASRAAAAAP
ZmWRKY53.1      KGNPNPRSYYKCTTAGCPVRKHVERASHDKRAVITTYEGKHNHDVPVGRGAASRAAAAAA
OsWRKY53        KGNPNPRSYYKCTTVGCPVRKHVERASHDTRAVITTYEGKHNHDVPVGRGGGGGRAPAPA
TaWRKY53        KGNPNPRSYYKCTTVGCPVRKHVERASHDNRAVITTYEGKHSHDVPIGRGRALPASSSS-
AtWRKY33        KGNPNPRSYYKCTTIGCPVRKHVERASHDMRAVITTYEGKHNHDVPAARGSGYATNRAPQ
                ************** ***********.** ***********.**** .** .     :

ZmWRKY53        L------LGSGGGQMD---HRHQQPYTLEMLSGGGG-----------------------G
ZmWRKY53.1      AAGSGALMATGGGQLGYHHQQQQQPYTLEMLSSGSY-----------------------G
OsWRKY53        PPTSGAIRP------SAVAAAQQGPYTLEMLPNPAGLYGGYGAG---------------A
TaWRKY53        ----------DSSAVIWPAAAVQAPCTLEMLAGHPG------------------------
AtWRKY33        DSSSVPIRPAAIAGHSNYTTSSQAPYTLQMLHNNNTNTGPFGYAMNNNNNNSNLQTQQNF
                                  * * **:**

ZmWRKY53        YGGGYA-AKDEPRDD-LFVDSLLC
ZmWRKY53.1      GGGGYAAAKDEPRDD-LFVDSLLC
OsWRKY53        GGAAFPRTKDERRDD-LFVESLLC
TaWRKY53        ----Y-AAKDEPRDD-MFVESLLC
AtWRKY33        VGGGFSRAKEEPNEETSFFDSFMP
                    :  :*:* .::  *.:*::
```

If *Zm*WRKY53 is regulated by MPK3 and MPK6 as observed for *At*WRKY33, this maize homolog may also promote the expression of ACC synthase genes, such as ASC2 and ASC6, which are involved in ethylene biosynthesis (Li et al. 2012). This would indicate that *Zm*WRKY53 may promote ethylene production and subsequent activation of ethylene-based defenses in resonse to *A. flavus* infection. In addition, a more recent study by Li et al. (2013) found that *ZmWRKY53*, which they designated as *ZmWRKY33*, was found to enhance salt stress tolerance when overexpressed in *A. thaliana* and was induced in response to cold, drought, and salt stress as well as by ABA application. This would also suggest that *Zm*WRKY53 may serve a dual role in promoting defenses against abiotic stress as well as pathogen infection.

The other candidate WRKY gene examined in this study, *ZmWRKY68* (MZ00042391), has homology to *At*WRKY11 (NP_849559.1) and *Os*WRKY68 (NP_001053792.1). Journot-Catalino et al. (2006) reported that *AtWRKY11* has partial functional redundancy to *AtWRKY17*, and that both genes function to promote the expression of JA-based defenses while suppressing SA-based defenses, although they also report that, for certain genes, *At*WRKY11 is a negative regulator of *At*WRKY17 due to an increase in *AtWRKY17* expression in response to silencing of *AtWRKY11*. However, both *Os*WRKY68 and *At*WRKY11 have been found to be homologous to the common grape vine (*Vitis vinifera*) WRKY11 (Liu et al. 2011), which was found to enhance resistance to *Pseudomonas syringae*, increase manitol-induced water stress tolerance, and enhance the expression of the stress response genes *AtRD29A* and *AtRD29B* when *VvWRKY11* is expressed in *A. thaliana*. They also found that *Vv*WRKY11 expression could also be rapidly induced by application of SA. In the present study, however, the expression of *ZmWRKY68* seems to indicate functional similarity with *VvWRKY11* given the suppression of expression observed in response to wounding in the resistant variety (Figure 4.5; Graph B),

a treatment which typically induces JA- and ethylene-based defenses (Ankala et al. 2009; Howe and Jander, 2008).

The final WRKY transcription factor examined in this study, PTZm631 (MZ00048386), which showed homology to *Zm*WRKY67 (BLASTx: Coverage = 71%, ID = 97%), was also found to have significant variation in expression patterns between the two varieties. In the susceptible line B73 (Figure 4.7; Graph A), *ZmWRKY67* was found to be heavily induced by inoculation early in the time course, reaching a maximum level at 4 DAI. Its expression in TZAR101 was induced by inoculation early from 2 to 4 DAI, but not to the same degree as in B73. A similar induction of this gene was noted in a previous study by Han et al. (2010, unpublished data) in which *ZmWRKY67* expression levels peaked at 4 DAI in the susceptible maize hybrid N83-N5 (Syngenta) in response to *A. flavus* inoculation.

A database search using BLASTp indicated that *Zm*WRKY67 is homologous to *At*WRKY50 (NP_197989.2). This gene was found to suppress JA-based defenses and promote SA-based defenses in response to reduced levels of oleic acid (18:1; Gao et al. 2011). In addition, Gao et al. (2009) found that silencing the gene encoding maize lipoxygenase 3 (*Zm*LOX3) resulted in an increase in oleic acid content, JA accumulation, and aflatoxin contamination. Also, studies performed by Severns et al. (2003) found that maize varieties with kernels containing high oil levels were more heavily contaminated with aflatoxin than those with lower levels of oil (Maggio-Hall et al. 2005). Zeringue et al. (1996) also observed that a higher ratio of linoleic acid (18:2) to oleic acid, and therefore lower levels of oleic acid, correlated to resistance to aflatoxin contamination in maize kernels. High oleic acid content has also been reported to correlate with susceptibility to aflatoxin contamination in peanut (Gao and Kolomiets, 2009; Xue et al. 2003). Since reduced oleic acid content correlates with reduced

aflatoxin levels in infected kernels and induction of *AtWRKY50* expression, *Zm*WRKY67 may function to suppress JA-based defenses and promote SA-based defenses later in infection in response to lower levels of oleic acid accumulation in the resistant line, while elevated expression early in infection in susceptible lines in response to infection may suppress initial JA-based defense gene expression.

5.2 Phytohormone Signaling Pathways

In addition, we also examined the expression of three pathway indicator genes to determine which major defense pathways are induced upon *A. flavus* inoculation of immature maize kernels. Two genes known to be induced as a part of SA-based defense responses, *ZmPR-1* and *ZmNPR1* (Spoel et al. 2007; Zhang et al. 1999), and one gene known to be induced by both JA- and ethylene-based defense responses, *ZmERF1* (Lorenzo et al. 2003), were examined. The expression of both *ZmPR-1* (Figure 4.8) and *ZmNPR1* (Figure 4.9) was suppressed by inoculation and wounding at earlier time points in comparison to the levels observed during normal development in B73. *ZmNPR1* expression was also found to be suppressed and induced by wounding at 6 DAI and 10 DAI, respectively, with no corresponding changes in *ZmPR-1* expression. In TZAR101, the expression of *ZmPR-1* did not significantly vary over the time course except at 14 DAI where a significant induction in response to inoculation and a slight suppression in response to wounding. The expression of *ZmNPR1*, however, did not show suppression as observed in B73.

Homologs of *Zm*PR-1 and *Zm*NPR1 in *A. thaliana* have been shown to respond to SA-mediated defense signals (Zhang et al. 1999). In addition, *At*NPR1 has been shown to function upstream of *AtPR-1* and regulates its expression indirectly through interaction with TGA transcription factors (Pieterse and van Loon, 2004). The expression of defense genes such as

AtPR-1 through *At*NPR1 mediated mechanisms is essential to the induction of systemic required resistance (SAR) in response to invading pathogens, particularly biotrophic plant pathogens (Glazebrook, 2005). In this study, we observed that, despite highly elevated levels of *ZmNPR1* expression in the resistant variety, TZAR101, there was no induction in *ZmPR-1* expression as expected. This may be due to varied expression of intermediate components in the pathway required for the expression of PR proteins, such as TGA transcription factors. TGA transcription factors are utilized for both SA and JA-induced defenses upstream of PR-1and may be responsible for regulating the activation of appropriate pathways (Kesarwani et al. 2007; Windram et al. 2012; Zander et al. 2010). *Zm*NPR1 activity may also be regulated in the nucleus by proteosome-mediated degradation after *Zm*NPR1 is tagged with ubiquitin by the CUL3 ubiquitin ligase along with the maize homologs of the adaptor proteins *At*NPR3 and *At*NPR4 (Fu et al. 2012; Pintard et al. 2004; Spoel et al. 2009).

In *A. thaliana*, *AtERF1* has been shown to be induced by both JA and ethylene (Lorenzo et al. 2003). Because of this, *At*ERF1 functions as an intersection of the JA and ethylene signaling pathways and is responsible for the regulation of several downstream genes such as defensin (PDF1.2) (Berrocal-Lobo et al. 2002; Lorenzo et al. 2003). Induced expression of *AtERF1* upon infection by necrotrophic pathogens, such as *Botrytis cinerea*, has been noted, as well as the conference of enhanced resistance to such pathogens by overexpression of this gene (Berrocal-Lobo et al. 2002; Berrocal-Lobo and Molina, 2004). In the present study, it was found that the *AtERF1* homolog, *ZmERF1*, was expressed at higher levels in the resistant variety, TZAR101, constitutively and in response to inoculation with *A. flavus*, than the susceptible variety, B73. This indicates that stimulation of JA or ethylene-based defenses in response to infection may play an important role in resistance, a characteristic of resistance to necrotrophic

pathogens (Glazebrook, 2005; Lorenzo et al. 2003). These findings concur with the results of microscopic studies of the host-pathogen interaction performed by Smart et al. (1990) which showed that *A. flavus* killed cells in advance of extending hyphae during infection of maize kernels indicating that *A. flavus* is a necrotrophic pathogen (Mideros et al. 2009). For both the pathway indicator genes and the examined WRKY transcription factor genes, the major patterns observed in their expression levels and putative functions of their respective orthologs in various species are summarized in Table 5.1.

5.3 Integration of WRKY Transcription Factors and Phytohormone Signaling

In contrast the concept of *A. flavus* as a necrotrophic pathogen, the expression levels of negative regulators of JA-based defenses were found to be induced in the resistant variety, though at later time points. In response to inoculation, *ZmWRKY67*, the homolog of *AtWRKY50*, was found to be expressed at increasing levels in the resistant variety over time, and, conversely, at higher levels at earlier time points in the susceptible variety which decreased over time. This may correlate with a lower accumulation of oleic (18:1) acid in resistant maize kernels (Zeringue et al. 1996), a condition which has been shown to induce the expression of *AtWRKY50* and *AtWRKY51* resulting in downstream induction of SA-responsive genes and the suppression of JA-responsive genes in *A. thaliana* (Gao et al. 2011). Therefore, *Zm*WRKY67 may function in a similar fashion as AtWRKY50/51 during *A. flavus* infection (Figure 5.2; Diagram A). Also, since oleic acid is a precursor in JA biosynthesis (Heldt and Piechulla, 2010; León and Sánchez-Serrano, 1999), the reduction in oleic acid may be due to the production of JA with no subsequent synthesis of replacement oleic acid, and may result in lower levels of JA and oleic acid in the resistant maize kernels during maturation. This is in agreement with previous studies which found that oleic acid accumulation in maize kernels is associated with susceptibility to

Table 5.1 Summary of gene expression analyses of maize WRKY transcription factors and pathway indicator genes.

Gene	Accession*	Observed Expression Pattern	Reported Ortholog Function	References
***Zm* WRKY19** (MZ00019797)	AFW81188.1	Induced by infection early, but expressed at higher levels in the resistant variety at later time points.	Calmodulin Interaction, MAP-Kinase Regulated, Oxidative Stress Response, Antioxidant Enzyme Promotion	Eulgem and Somssich, 2007; Miao et al. 2004, 2007; Popescu et al. 2007; Rushton et al. 2010
***Zm* WRKY21** (MZ00026377)	NP_001150830.1	No significant variation in expression in either the resistant or susceptible variety.	Calmodulin Interaction, H_2O_2-Induced, Potentially Ethylene-Regulated, Thermotolerance	Hass et al. 2004; Li et al. 2010; Park et al. 2005; Popescu et al. 2007; Vanderauwera et al. 2005
***Zm* WRKY53** (MZ00021479)	NP_001147949.1	Induced by infection, yet earlier in the resistant variety than in the susceptible variety.	ABA Responsive, Ethylene Biosynthesis, MAP-Kinase Regulated, Necrotrophic Resistance, Phytoalexin Production, PR-Gene Regulation	Andreasson et al. 2005; Birkenbihl and Somssich, 2011; Birkenbihl et al.2012; Chujo et al. 2007; Eulgem and Somssich, 2007; Kishi-Kaboshi et al. 2010; Li et al. 2012, 2013; Mao et al. 2011; Qiu et al. 2008; Rushton et al. 2010; van Eck, 2011; van Eck et al. 2010; Yu et al. 2010
***Zm* WRKY53.1** (MZ00042508)	NP_001147551.1	Much higher expression in the susceptible variety than in the resistant variety at all time points under both control and inoculation conditions.		
***Zm* WRKY67** (PTZm631)	NP_001148599.1	Induced by infection early in both varieties, but constitutively and inducibly higher in response to infection at later timepoints in the resistant variety.	Early Induction in a Susceptible Maize Hybrid; SA-Based Defense Induction in Response to Low Oleic Acid	Gao et al. 2011; Han et al. 2010
***Zm* WRKY68** (MZ00042391)	ACG45417.1	Variable expression in response to treatments; Suppressed in response to wounding in the resistant variety.	Hemibiotroph Resistance, Ethylene and JA-Based Defense Gene Expression, Water-Stress Tolerance	Ankala et al. 2009; Howe and Jander, 2008; Journot-Catalino et al. 2006; Liu et al. 2011
***Zm* ERF-1**	NP_001105270.1	Higher constitutive levels of expression in the resistant variety than in the susceptible variety.	PR-Gene Regulation; Responsive to Ethylene and JA Defense Signalling	Berrocal-Lobo and Molina, 2004; Berrocal-Lobo et al. 2002; Lorenzo et al. 2003; Takeuchi et al. 2011; Van der Does et al. 2013
***Zm* NPR-1**	ACG45791.1	Early suppression of expression in response to wounding in the susceptible variety; Induced by infection and expressed at constitutively higher levels in the resistant variety, though suppressed at later timepoints by wounding.	Biotroph Resistance; Regulation of WRKY Transcription Factor Expression; Responsive to SA Defense Signalling	Glazebrook, 2005; Fu et al. 2012; Pieterse and van Loon, 2004; Pintard et al. 2004; Spoel et al. 2007, 2009; Zhang et al. 1999
***Zm* PR-1**	NP_001152581.1	Early suppression of expression in response to wounding in the susceptible variety; No significant variation in expression in the resistant variety except at 14 DAI.	Biotroph Resistance; Responsive to SA Defense Signalling	Glazebrook, 2005; Kesarwani et al. 2007; Pieterse and van Loon, 2004; Spoel et al. 2007; Windram et al. 2012; Zander et al. 2010; Zhang et al. 1999

*NCBI Genebank accession of gene product.

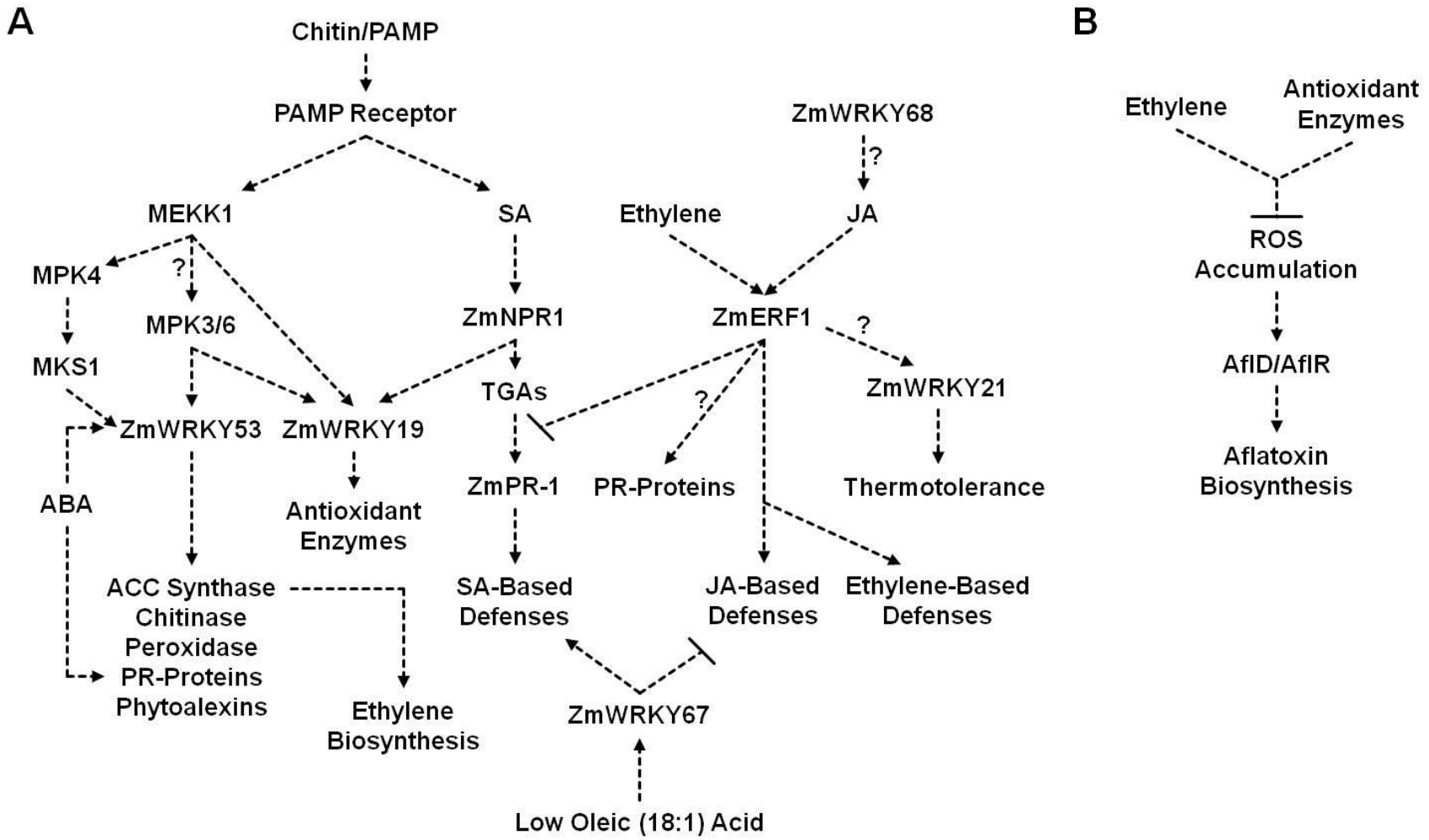

Figure 5.2 Putative pathway of defense gene activation in resistant maize lines. A: The putative pathway of defense gene activation in immature maize kernel tissue following inoculation with *Aspergillus flavus*. B: The possible effects of ethylene and antioxidant enzyme accumulation on the production of aflatoxin in *A. flavus* during infection.

aflatoxin contamination (Gao et al. 2009; Zeringue et al. 1996). Other studies have also reported that methyl jasmonate (MeJA) increased levels of aflatoxin production in *A. flavus* depending on culture conditions (Goodrich-Tanrikulu et al. 1995; Meimaroglou et al. 2009; Vergopoulou et al. 2001).

With regard to SA-regulated defenses, Magbanua et al. (2007) observed that there was a greater accumulation of SA in resistant varieties in comparison to susceptible varieties. This, taken in light of the present study, may indicate that greater SA accumulation in the resistant line may be the cause of the increase in expression of the upstream, SA-responsive gene, *ZmNPR1* (Zhang et al. 1999). These findings, though, somewhat conflicting with the convention that JA promotes defense against necrotrophic pathogens (Glazebrook, 2005). However, a recent study by Van der Does et al. (2013) found that the application of SA does not significantly affect the expression of *AtERF1*, but does suppress the expression of the transcription factor octadecanoid-responsive *Arabidopsis* AP2/ERF-domain protein 59 (ORA59) leading to the suppression of JA-induced defense genes. Given these findings and the elevated level of expression of *ZmERF1* observed in this study in the resistant variety, ethylene-based defense components may also be active at this time and may partially suppress the expression of *ZmPR-1* downstream of *Zm*NPR1 through the regulation of TGA transcription factor expression (Kesarwani et al. 2007; Windram et al. 2012; Zander et al. 2010). Therefore, this accumulation of SA in resistant varieties as suggested by Magbanua et al. (2007), though not resulting in *Zm*NPR1-mediated defense gene activation, such as in the case of *Zm*PR-1, may contribute to resistance in an alternative manner.

Increases in SA have been found to result in the accumulation of reactive oxygen species (ROS), such as H_2O_2, leading to the stimulation of ROS sequestering enzyme activity (Larkindale and Kinght, 2002; Rao at al. 1997). It has been shown that *At*WRKY53 expression is

regulated either through the action of NPR1/TGA transcription factor complexes or independent of SA-regulated signaling mechanisms through MAPK pathway components, such as MEKK1, and can and it can bind to the promoters of genes encoding antioxidant enzymes including several isoforms of catalase (Eulgem and Somssich, 2007; Miao et al. 2004, 2007; Rushton et al. 2010). If *Zm*WRKY19, the homolog of *At*WRKY53, functions in a similar manner, it is possible that resistant varieties may display greater antioxidant enzyme accumulation and activity to a greater extent than susceptible lines, allowing for enhanced tolerance of SA or pathogen induced ROS accumulation. This may be plausible considering that such increases have been reported in resistant maize lines (Chen et al. 2012a; Magbanua et al. 2007; Pechanova et al. 2011).

The accumulation of antioxidant proteins may also be due to the function of *Zm*WRKY53 and the signaling pathway mediated through MPK3 and MPK6, which have been implicated in the production of ROS in hypersensitivity induced cell death, and chitin perception independent of phytohormone signalling pathways (Liu et al. 2007; Mao et al. 2011; Wan et al. 2004; Zhang et al. 2002).The wheat homolog of *Zm*WRKY53, *Ta*WRKY53, has also been shown to interact with the antioxidant enzyme GST (Gill and Tuteja, 2010; van Eck, 2011), and both the wheat and rice homologs of *Zm*WRKY53, *Ta*WRKY53 and *Os*WRKY53, have been found to potentially regulate the expression of chitinases, peroxidases, and various PR genes (Chujo et al. 2007; van Eck, 2011). Therefore, both *Zm*WRKY19 and *Zm*WRKY53 may function independently of SA, JA, or ethylene-mediated signaling pathways through MAP Kinase to confer both ROS tolerance and resistance to *A. flavus* (Figure 5.2; Diagram A).

The potential increase in antioxidant enzyme levels in response to elevated *Zm*WRKY19 and *Zm*WRKY53 levels in resistant varieties has four potential implications. First, increased accumulation of these proteins in a constitutive fashion may confer resistance to abiotic stresses

that cause negative effects through the production of ROS, such as drought or heat stress (Hu et al. 2008, 2010; Volkov et al. 2006). This may explain the apparent correlation between abiotic stress tolerance and *A. flavus* resistance (Kebede et al. 2012; Pechanova et al. 2011). Second, constitutive accumulation of these proteins may result in the prevention of host cell death caused by the necrotroph-induced accumulation of ROS in colonized tissue, a potential mechanism for resistance to the necrotroph, *A. flavus* (Glazebrook, 2005; Mideros et al. 2009; Smart et al. 1990). Third, the increase in antioxidant enzyme levels, combined with an increase in SA levels, may lead to a change in cytoplasmic reduction potential resulting in the breakdown of NPR1 oligomers into individual monomers facilitating their translocation into the nucleus, as observed in *A. thaliana* (Brosché and Kangasjärvi, 2012; Mou et al. 2003; Peleg-Grossman et al. 2010). Finally, this reduction in ROS, in conjunction with the potential increase in ethylene production as indicated by increased *ZmERF1* expression in the resistant variety, may cause reduced oxidative stress on *A. flavus* itself causing a reduction of *AflD* and *AflR* expression, which in turn lead to reduced aflatoxin accumulation in the resistant variety (Figure5.2; Diagram B), (Huang et al. 2009).

In addition, increased *ZmERF1* expression may result in the accumulation in various PR genes, such as *ZmPR10* or *ZmPR10.1* (Chen et al. 2006, 2010; Xie et al. 2010), since recent studies have indicated that *Os*ERF1 regulates the expression of the root specific PR10 gene, *RSOsPR10* (Takeuchi et al. 2011). This may be plausible since the expression of genes encoding these isoforms of *Zm*PR10-like proteins has been shown to be induced in maize lines resistant to *A. flavus* infection and aflatoxin accumulation (Chen et al., 2010), and *ZmERF1* was found to be expressed at a higher level in the resistant variety, TZAR101, in the present study. A conserved domain search (Marchler-Bauer et al. 2013) of the amino acid sequences of both *Zm*PR10

(AAY29574.1) and *Zm*PR10.1 (ADA68331.1) revealed that they both possess PYR/PYL/RCAR-like domains which function in ABA signal transduction (Melcher et al. 2010; Nishimura et al. 2010; Santiago et al. 2009; Yin et al. 2009). Since *ZmWRKY53* was also recently found to be induced in response to ABA application (Li et al. 2013), it is possible that ABA can modulate the expression of key defense components in *A. flavus* resistance, a trend which has been observed in previous studies under drought conditions (Jiang et al. 2012). Additional studies of the interaction of these factors may allow for a possible explanation for the correlation of *A. flavus* resistance and abiotic stress tolerance (Kebede et al. 2012).

5.4 Conclusions and Suggestions for Future Research

Contamination of maize kernels with aflatoxins resulted from pre- and post-harvest colonization of *Aspergillus flavus* causes not only economic losses due to crop lost, but also poses a serious threat to human and animal health (Payne and Widstrom, 1992; Schmale and Munkvold, 2011; Shephard 2008). To date, however, no single protein or gene has been identified as a major source of resistance in any of the aflatoxin-resistant maize genotypes. In addition, the mechanism of how genes regulating defense responses are controlled has not been well understood. Therefore, a better understanding of the molecular signaling pathways and the transcription factors involved in the promotion, suppression, or overall regulation of defense gene expression is essential.

In the present study, we identified that the expression of several maize WRKY transcription factors and pathway indicator genes. We found that several WRKY transcription factor-coding genes were significantly regulated by *A. flavus* inoculation. These WRKY transcription factors, particularly *Zm*WRKY53, in conjunction with stimulation of phytohormone signaling pathways, may play key roles in the regulation of defenses in response to *A. flavus*

infection and subsequent aflatoxin accumulation. In addition, *Zm*WRKY53.1, which was consistently expressed at significantly higher levels in the susceptible line than the resistant one at either control or under *A. flavus* inoculation conditions, may be a potential marker to be used in future marker assisted breeding studies to enhance maize aflatoxin resistance. However, further studies examining the precise functional characteristics of these WRKY transcription factors will be necessary given the limitations of the present study. For both the WRKY transcription factor coding genes and the pathway component genes, a high degree of variation was noted in the expression levels over the course of the study. This may be due to environmental influences as well as the present study's use of whole kernel tissues. To remedy this, future studies should examine the expression of these selected WRKY transcription factors in individual kernel tissues, particularly embryo tissue where clearer treatment effects and higher expression levels may be observed. In addition, wounded controls in future studies should also be applied by injecting sterile inoculum base, in this instance 0.01% tween 20 buffer, to examine any possible effects of tween 20 on the expression of maize WRKY transcription factors.

In addition, since the present study does not examine the proteomic responses of the examined maize varieties, and several of the WRKY transcription factors examined in this study including *Zm*WRKY19, -21, -67, and -68 have yet to be fully characterized at the protein level, future studies of their expression at protein level are encouraged in order to better understand the precise functions of the examined WRKY transcription factors following *A. flavus* inoculation. These functional analyses and, especially, the quantification of the levels of individual WRKY proteins using proteomics approaches are critical given the potential for a lack of correlation of transcript and protein levels in the host tissues. The identification of the promoter regions in the maize genome targeted by these WRKYs is also needed to validate the proposed model and

could be performed using chromatin immunoprecipitation analyses (ChIP) (Abdalla et al. 2009; Weinmann and Farnham, 2002; Wyrick and Young, 2002). The effect of aflatoxin biosynthesis or *A. flavus* strain on the expression of WRKY transcription factors could also be examined by infecting maize with toxigenic and atoxigenic strains of the fungus. Additional WRKY transcription factors should also be examined since the initial selection of the candidate genes analyzed in the present study was based on data obtained by Luo et al. (2011) under KSA conditions which may result in the exclusion of other key WRKY genes that may be only expressed in immature kernels under field conditions.

Overall, however, the present study provides a basis on which to build a working model (Figure 5.2) for the partial regulation of *A. flavus* resistance. It also provides a framework from which to design and plan future experiments. By better understanding these regulatory mechanisms through transcript and protein level analyses, additional tools will be made available for incorporation through breeding and genetic engineering to improve maize resistance to *A. flavus* and aflatoxin contamination.

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APPENDIX A
CONTRASTS OF TYPE III FIXED EFFECTS FOR *Zm*WRKY TRANSCRIPTION FACTOR AND PATHWAY GENE RELATIVE EXPRESSION ANALYSES

Table A.1 Contrasts of type III fixed effects for the putative *ZmWRKY19* (MZ00019797) gene relative expression levels.

Label	Numerator dF	Denominator dF	F Value	Pr > F
VAR	1	5.48	11.07	0.0181
TNT	2	217	9.82	<.0001
VAR*TNT	2	217	2.36	0.0973
DAI	8	28.2	1.14	0.3699
VAR*DAI	8	29.2	0.68	0.7082
TNT*DAI	16	215	3.18	<.0001
VAR*TNT*DAI	14	210	2.69	0.0012

Table A.2 Contrasts of type III fixed effects for the *ZmWRKY53* (MZ00021479) gene relative expression levels.

Label	Numerator dF	Denominator dF	F Value	Pr > F
VAR	1	5.87	5.14	0.0648
TNT	2	217	6.82	0.0013
VAR*TNT	2	218	1.67	0.1899
DAI	8	32.5	0.87	0.5502
VAR*DAI	8	33	0.59	0.7763
TNT*DAI	16	219	1.77	0.0361
VAR*TNT*DAI	14	216	2.36	0.0046

Table A.3 Contrasts of type III fixed effects for the *ZmWRKY21* (MZ00026377) gene relative expression levels.

Label	Numerator dF	Denominator dF	F Value	Pr > F
VAR	1	4.89	0.49	0.5153
TNT	2	224	5.29	0.0057
VAR*TNT	2	223	2.64	0.0733
DAI	8	40	4.61	0.0005
VAR*DAI	8	41.9	2.02	0.0681
TNT*DAI	16	223	2.16	0.0071
VAR*TNT*DAI	14	221	2.04	0.0159

Table A.4 Contrasts of type III fixed effects for the *ZmWRKY68* (MZ00042391) gene relative expression levels.

Label	Numerator dF	Denominator dF	F Value	Pr > F
VAR	1	5.07	0.15	0.7105
TNT	2	214	4.88	0.0084
VAR*TNT	2	217	4.89	0.0084
DAI	8	31.2	0.48	0.8587
VAR*DAI	8	31.9	2.01	0.0766
TNT*DAI	16	215	2.8	0.0004
VAR*TNT*DAI	14	212	2.93	0.0004

Table A.5 Contrasts of type III fixed effects for the *ZmWRKY53.1* (MZ00042508) gene relative expression levels.

Label	Numerator dF	Denominator dF	F Value	Pr > F
VAR	1	4.99	370.64	<.0001
TNT	2	222	4.18	0.0165
VAR*TNT	2	216	0.16	0.8525
DAI	8	27.7	1.79	0.1222
VAR*DAI	8	28.9	0.98	0.4714
TNT*DAI	16	218	2.91	0.0002
VAR*TNT*DAI	14	216	1.29	0.2174

Table A.6 Contrasts of type III fixed effects for the *ZmWRKY67* (PTZm631) gene relative expression levels.

Label	Numerator dF	Denominator dF	F Value	Pr > F
VAR	1	5.47	0.02	0.8995
TNT	2	218	19.84	<.0001
VAR*TNT	2	212	0.17	0.8426
DAI	8	31.4	3.78	0.0033
VAR*DAI	8	32.5	0.63	0.7451
TNT*DAI	16	219	2.25	0.0048
VAR*TNT*DAI	14	215	1.95	0.0225

Table A.7 Contrasts of type III fixed effects for the *ZmPR-1* gene relative expression levels.

Label	Numerator dF	Denominator dF	F Value	Pr > F
VAR	1	3.4	20.38	0.0155
TNT	2	124	3.91	0.0225
VAR*TNT	2	127	1.11	0.3315
DAI	7	21.4	2.92	0.0263
VAR*DAI	7	22.4	3.23	0.016
TNT*DAI	13	146	2.59	0.0029
VAR*TNT*DAI	11	147	1.15	0.3306

Table A.8 Contrasts of type III fixed effects for the *ZmNPR1* gene relative expression levels.

Label	Numerator dF	Denominator dF	F Value	Pr > F
VAR	1	4.03	30.56	0.0051
TNT	2	144	3.73	0.0264
VAR*TNT	2	146	5.65	0.0043
DAI	7	21.3	1.47	0.2324
VAR*DAI	7	22.2	0.93	0.5056
TNT*DAI	13	147	1.84	0.0414
VAR*TNT*DAI	11	149	1.45	0.1569

Table A.9 Contrasts of type III fixed effects for the *ZmERF1* gene relative expression levels.

Label	Numerator dF	Denominator dF	F Value	Pr > F
VAR	1	3.05	3.61	0.1522
TNT	2	155	2.27	0.1067
VAR*TNT	2	156	6.66	0.0017
DAI	7	22.1	4.45	0.0032
VAR*DAI	7	23.2	2.57	0.041
TNT*DAI	13	142	1.56	0.1034
VAR*TNT*DAI	11	148	1.73	0.0729

APPENDIX B
TABULAR PRESENTATION OF DATA FOUND IN FIGURES

This appendix contains tabular forms of the data which appear in figures. Table B.1 contains the aflatoxin data obtained for the varieties examined in the present study during a field screening assay conducted in 2012. The varieties B73 (susceptible) and TZAR101 (resistant) were inoculated with a conidial suspension with a concentration 4.0×10^6 conidia/mL in a 0.01% tween 20 buffer, wounded to simulate the physical injury incurred during inoculation, or non-treated. At kernel maturity (~ 60 DAP, seed moisture level < 15%), aflatoxin analysis of kernels surrounding the inoculation sites, approximately 20g, were performed by Dr. Robert L. Brown (USDA-ARS: SRRC) using an AgraQuant total aflatoxin ELISA test kit (Romer Labs, Union, MO, USA) according to the manufacturer's instructions (Zheng et al. 2005).The reported values in Table B.1 are the average aflatoxin levels (ppb) ± standard error. Statistical groupings are based on Tukey's LSD analysis with $\alpha = 0.05$.

Table B.2 through Table B.10 contain tabular forms of the data which appear in figures displaying the relative expression data for the examined WRKY transcription factors and the pathway indicator genes. The relative expression levels of these genes over the time course in the resistant and susceptible varieties in response to treatments were determined using qPCR analysis. The data present in these tables are the average relative expression values for the three-way interactions between variety x treatment x DAI ± standard error. The values were obtained using the equation relative gene expression = $[(E+1)^{(\text{Ct } Zm18\text{S rRNA})} / (E+1)^{(\text{Ct target gene})}]$, where E is derived from the equation $E = 10^{(-1/\text{Slope})} - 1$, followed by data transformation performed by multiplying the relative gene expression by 1.0×10^9 followed by natural logarithm transformation (Chen et al. 2010; Jiang et al. 2012; Pfaffl, 2001).

Table B.1 Average aflatoxin accumulation in maize lines B73 and TZAR101 during the 2012 field assay (Tabular presentation of data found in Figure 4.1).

Variety	Treatment	Average ± Std. Error[1]	Group[2]
B73	Non-Treated Control	12.5171 ± 115.2	B
	Wounded	10.8667 ± 131.73	B
	Infected	2042.49 ± 122.64	A
TZAR101	Non-Treated Control	1.5623 ± 86.7167	B
	Wounded	4.8543 ± 99.0375	B
	Infected	202.98 ± 89.7604	B

[1]Values represent the average aflatoxin levels in ppb ± standard error.

[2]Statistical grouping based on Tukey's LSD analysis, $\alpha = 0.05$.

Table B.2 Relative expression levels of the *ZmWRKY19* gene (Tabular presentation of data found in Figure 4.2).

Variety	Treatment	DAI	Average RE ± Std. Error
B73	NTC	0	3.4769 ± 0.7098
B73	NTC	1	3.0441 ± 0.6911
B73	NTC	2	1.905 ± 0.847
B73	NTC	3	3.9838 ± 0.9058
B73	NTC	4	1.5244 ± 0.824
B73	NTC	6	2.5845 ± 0.6924
B73	NTC	10	2.3484 ± 0.7235
B73	NTC	14	2.6675 ± 0.7046
B73	NTC	18	4.8867 ± 0.849
B73	Infected	0	3.4769 ± 0.7098
B73	Infected	1	4.3788 ± 0.6209
B73	Infected	2	4.6506 ± 0.739
B73	Infected	3	2.6851 ± 0.9058
B73	Infected	4	4.8174 ± 0.6661
B73	Infected	6	3.1953 ± 0.626
B73	Infected	10	3.226 ± 0.621
B73	Infected	14	2.8724 ± 0.636
B73	Infected	18	3.127 ± 0.6827
B73	Wounded	0	3.4769 ± 0.7098
B73	Wounded	1	2.7866 ± 0.6209
B73	Wounded	2	3.7009 ± 0.7401
B73	Wounded	3	3.7262 ± 0.9058
B73	Wounded	4	3.5154 ± 0.6576
B73	Wounded	6	3.0386 ± 0.626
B73	Wounded	10	2.9554 ± 0.6509
B73	Wounded	14	3.2876 ± 0.9868
TZAR101	NTC	0	3.784 ± 0.6149
TZAR101	NTC	1	3.7075 ± 0.6372
TZAR101	NTC	2	4.2685 ± 0.9029
TZAR101	NTC	3	4.2011 ± 0.7324
TZAR101	NTC	4	4.4134 ± 0.8342
TZAR101	NTC	6	3.8766 ± 0.6507
TZAR101	NTC	10	4.9128 ± 0.6412
TZAR101	NTC	14	4.5806 ± 0.6426
TZAR101	NTC	18	4.6453 ± 0.6606
TZAR101	Infected	0	3.784 ± 0.6149
TZAR101	Infected	1	4.9276 ± 0.5941
TZAR101	Infected	2	4.3254 ± 1.0308
TZAR101	Infected	3	4.3779 ± 0.7324
TZAR101	Infected	4	4.9476 ± 0.6526
TZAR101	Infected	6	4.0632 ± 0.5941
TZAR101	Infected	10	4.7618 ± 0.5942
TZAR101	Infected	14	4.8038 ± 0.6
TZAR101	Infected	18	5.0369 ± 0.6606
TZAR101	Wounded	0	3.784 ± 0.6149
TZAR101	Wounded	1	4.2324 ± 0.6174
TZAR101	Wounded	3	3.8744 ± 0.7324
TZAR101	Wounded	4	3.8246 ± 0.6808
TZAR101	Wounded	6	3.9372 ± 0.613
TZAR101	Wounded	10	3.7601 ± 0.7228
TZAR101	Wounded	14	3.7816 ± 0.6778
TZAR101	Wounded	18	4.4284 ± 0.8585

Table B.3 Relative expression levels of the *ZmWRKY53* gene (Tabular presentation of data found in Figure 4.3).

Variety	Treatment	DAI	Average RE ± Std. Error
B73	NTC	0	3.7948 ± 0.4171
B73	NTC	1	4.115 ± 0.4033
B73	NTC	2	3.9122 ± 0.574
B73	NTC	3	4.3755 ± 0.6207
B73	NTC	4	3.4219 ± 0.5485
B73	NTC	6	3.3011 ± 0.4048
B73	NTC	10	2.7684 ± 0.4436
B73	NTC	14	3.5428 ± 0.4191
B73	NTC	18	4.2818 ± 0.5762
B73	Infected	0	3.7948 ± 0.4171
B73	Infected	1	4.2834 ± 0.3136
B73	Infected	2	4.1143 ± 0.456
B73	Infected	3	3.3971 ± 0.6207
B73	Infected	4	4.8597 ± 0.3709
B73	Infected	6	3.8038 ± 0.3206
B73	Infected	10	3.8241 ± 0.3134
B73	Infected	14	3.2943 ± 0.3345
B73	Infected	18	3.233 ± 0.3928
B73	Wounded	0	3.7948 ± 0.4171
B73	Wounded	1	3.3305 ± 0.3132
B73	Wounded	2	3.9634 ± 0.456
B73	Wounded	3	3.7118 ± 0.6207
B73	Wounded	4	3.7901 ± 0.3597
B73	Wounded	6	3.3557 ± 0.3206
B73	Wounded	10	3.8972 ± 0.3545
B73	Wounded	14	3.0517 ± 0.7146
TZAR101	NTC	0	3.8147 ± 0.3087
TZAR101	NTC	1	3.8173 ± 0.3393
TZAR101	NTC	2	4.1217 ± 0.6185
TZAR101	NTC	3	3.6329 ± 0.45
TZAR101	NTC	4	3.9438 ± 0.561
TZAR101	NTC	6	4.0347 ± 0.3583
TZAR101	NTC	10	4.6126 ± 0.3442
TZAR101	NTC	14	3.9422 ± 0.3466
TZAR101	NTC	18	4.4463 ± 0.3661
TZAR101	Infected	0	3.8147 ± 0.3087
TZAR101	Infected	1	4.7463 ± 0.2792
TZAR101	Infected	2	5.497 ± 0.7487
TZAR101	Infected	3	4.3024 ± 0.45
TZAR101	Infected	4	4.8725 ± 0.3556
TZAR101	Infected	6	3.712 ± 0.2792
TZAR101	Infected	10	4.2816 ± 0.2793
TZAR101	Infected	14	4.9137 ± 0.2884
TZAR101	Infected	18	4.7064 ± 0.3661
TZAR101	Wounded	0	3.8147 ± 0.3087
TZAR101	Wounded	1	4.2896 ± 0.3137
TZAR101	Wounded	3	4.3076 ± 0.45
TZAR101	Wounded	4	3.9678 ± 0.3917
TZAR101	Wounded	6	4.0428 ± 0.3073
TZAR101	Wounded	10	3.9936 ± 0.446
TZAR101	Wounded	14	3.9711 ± 0.3915
TZAR101	Wounded	18	4.2021 ± 0.5729

Table B.4 Relative expression levels of the *ZmWRKY21* gene (Tabular presentation of data found in Figure 4.4).

Variety	Treatment	DAI	Average RE ± Std. Error
B73	NTC	0	5.4143 ± 0.87
B73	NTC	1	4.7061 ± 0.8599
B73	NTC	2	3.3018 ± 0.9276
B73	NTC	3	6.0754 ± 0.9578
B73	NTC	4	3.2735 ± 0.9145
B73	NTC	6	4.0651 ± 0.8604
B73	NTC	10	3.4714 ± 0.8733
B73	NTC	14	3.5507 ± 0.8662
B73	NTC	18	3.2215 ± 0.9277
B73	Infected	0	5.4143 ± 0.87
B73	Infected	1	3.6924 ± 0.8337
B73	Infected	2	3.605 ± 0.8812
B73	Infected	3	3.4978 ± 0.9578
B73	Infected	4	3.5255 ± 0.851
B73	Infected	6	3.7253 ± 0.8355
B73	Infected	10	3.5501 ± 0.8337
B73	Infected	14	3.4962 ± 0.8393
B73	Infected	18	3.1615 ± 0.8576
B73	Wounded	0	5.4143 ± 0.87
B73	Wounded	1	3.8526 ± 0.8337
B73	Wounded	2	3.66 ± 0.8812
B73	Wounded	3	4.1152 ± 0.9578
B73	Wounded	4	4.2051 ± 0.8478
B73	Wounded	6	3.6196 ± 0.8356
B73	Wounded	10	3.3255 ± 0.8448
B73	Wounded	14	3.6939 ± 0.9924
TZAR101	NTC	0	3.9779 ± 0.8319
TZAR101	NTC	1	4.2185 ± 0.8395
TZAR101	NTC	2	4.586 ± 0.9566
TZAR101	NTC	3	4.1651 ± 0.8789
TZAR101	NTC	4	3.9969 ± 0.9214
TZAR101	NTC	6	3.778 ± 0.8447
TZAR101	NTC	10	3.7332 ± 0.841
TZAR101	NTC	14	3.7903 ± 0.8417
TZAR101	NTC	18	3.6885 ± 0.8494
TZAR101	Infected	0	3.9779 ± 0.8319
TZAR101	Infected	1	3.8455 ± 0.824
TZAR101	Infected	2	3.498 ± 1.0182
TZAR101	Infected	3	3.7261 ± 0.8789
TZAR101	Infected	4	4.1542 ± 0.8463
TZAR101	Infected	6	3.7931 ± 0.824
TZAR101	Infected	10	3.8831 ± 0.8241
TZAR101	Infected	14	3.6468 ± 0.8261
TZAR101	Infected	18	3.6818 ± 0.8494
TZAR101	Wounded	0	3.9779 ± 0.8319
TZAR101	Wounded	1	4.0501 ± 0.8323
TZAR101	Wounded	3	3.9665 ± 0.8789
TZAR101	Wounded	4	3.6753 ± 0.8571
TZAR101	Wounded	6	3.7094 ± 0.8308
TZAR101	Wounded	10	3.3636 ± 0.873
TZAR101	Wounded	14	3.0547 ± 0.8551
TZAR101	Wounded	18	3.0667 ± 0.9368

Table B.5 Relative expression levels of the *ZmWRKY68* gene (Tabular presentation of data found in Figure 4.5).

Variety	Treatment	DAI	Average RE ± Std. Error
B73	NTC	0	4.6436 ± 0.7564
B73	NTC	1	5.2093 ± 0.7391
B73	NTC	2	3.9295 ± 0.8473
B73	NTC	3	5.917 ± 0.8956
B73	NTC	4	3.6364 ± 0.8257
B73	NTC	6	3.9104 ± 0.7408
B73	NTC	10	2.8923 ± 0.7606
B73	NTC	14	3.3903 ± 0.7493
B73	NTC	18	4.2647 ± 0.8471
B73	Infected	0	4.6436 ± 0.7564
B73	Infected	1	3.8809 ± 0.6966
B73	Infected	2	3.9433 ± 0.7758
B73	Infected	3	2.8141 ± 0.8956
B73	Infected	4	4.788 ± 0.7257
B73	Infected	6	3.7092 ± 0.6994
B73	Infected	10	3.6243 ± 0.6964
B73	Infected	14	3.1688 ± 0.7056
B73	Infected	18	3.3567 ± 0.7365
B73	Wounded	0	4.6436 ± 0.7564
B73	Wounded	1	3.7071 ± 0.6964
B73	Wounded	2	4.3353 ± 0.7758
B73	Wounded	3	3.2813 ± 0.8956
B73	Wounded	4	3.9847 ± 0.7208
B73	Wounded	6	3.4894 ± 0.6997
B73	Wounded	10	3.9744 ± 0.7148
B73	Wounded	14	3.4366 ± 0.9427
TZAR101	NTC	0	3.6807 ± 0.6879
TZAR101	NTC	1	3.7682 ± 0.7005
TZAR101	NTC	2	4.6916 ± 0.8898
TZAR101	NTC	3	3.9708 ± 0.7668
TZAR101	NTC	4	3.995 ± 0.8324
TZAR101	NTC	6	4.3572 ± 0.7088
TZAR101	NTC	10	4.3101 ± 0.7027
TZAR101	NTC	14	4.6751 ± 0.7041
TZAR101	NTC	18	4.5485 ± 0.7181
TZAR101	Infected	0	3.6807 ± 0.6879
TZAR101	Infected	1	3.9033 ± 0.6742
TZAR101	Infected	2	4.0419 ± 0.9791
TZAR101	Infected	3	3.8902 ± 0.7676
TZAR101	Infected	4	4.5757 ± 0.7127
TZAR101	Infected	6	4.1495 ± 0.6742
TZAR101	Infected	10	4.4743 ± 0.6745
TZAR101	Infected	14	4.6739 ± 0.6781
TZAR101	Infected	18	4.9613 ± 0.7185
TZAR101	Wounded	0	3.6807 ± 0.6879
TZAR101	Wounded	1	3.8682 ± 0.6882
TZAR101	Wounded	3	3.9695 ± 0.7668
TZAR101	Wounded	4	3.5453 ± 0.7308
TZAR101	Wounded	6	3.7868 ± 0.6858
TZAR101	Wounded	10	3.9353 ± 0.755
TZAR101	Wounded	14	3.2679 ± 0.7266
TZAR101	Wounded	18	3.7762 ± 0.8595

Table B.6 Relative expression levels of the *ZmWRKY53.1* gene (Tabular presentation of data found in Figure 4.6).

Variety	Treatment	DAI	Average RE ± Std. Error
B73	NTC	0	8.0883 ± 0.5206
B73	NTC	1	8.5159 ± 0.5068
B73	NTC	2	7.8892 ± 0.68
B73	NTC	3	8.4358 ± 0.7296
B73	NTC	4	7.6218 ± 0.6558
B73	NTC	6	7.5829 ± 0.51
B73	NTC	10	7.1713 ± 0.5478
B73	NTC	14	7.7107 ± 0.5224
B73	NTC	18	8.3581 ± 0.6847
B73	Infected	0	8.0883 ± 0.5206
B73	Infected	1	8.8423 ± 0.4182
B73	Infected	2	9.0537 ± 0.5546
B73	Infected	3	7.7235 ± 0.7319
B73	Infected	4	9.257 ± 0.4719
B73	Infected	6	8.2822 ± 0.4249
B73	Infected	10	7.9816 ± 0.4181
B73	Infected	14	7.6775 ± 0.4381
B73	Infected	18	7.8876 ± 0.4943
B73	Wounded	0	8.0883 ± 0.5206
B73	Wounded	1	7.8794 ± 0.4182
B73	Wounded	2	8.4068 ± 0.5546
B73	Wounded	3	8.492 ± 0.7319
B73	Wounded	4	8.1887 ± 0.4609
B73	Wounded	6	7.9918 ± 0.4257
B73	Wounded	10	8.1523 ± 0.4575
B73	Wounded	14	7.6959 ± 0.8358
TZAR101	NTC	0	3.1224 ± 0.4132
TZAR101	NTC	1	3.658 ± 0.4441
TZAR101	NTC	2	4.1679 ± 0.7279
TZAR101	NTC	3	3.8973 ± 0.5489
TZAR101	NTC	4	2.9404 ± 0.6708
TZAR101	NTC	6	3.4097 ± 0.4625
TZAR101	NTC	10	3.6632 ± 0.4488
TZAR101	NTC	14	3.24 ± 0.451
TZAR101	NTC	18	3.5148 ± 0.468
TZAR101	Infected	0	3.1224 ± 0.4132
TZAR101	Infected	1	3.8267 ± 0.3868
TZAR101	Infected	2	4.6715 ± 0.8697
TZAR101	Infected	3	3.5247 ± 0.5489
TZAR101	Infected	4	4.0489 ± 0.4576
TZAR101	Infected	6	2.8451 ± 0.3868
TZAR101	Infected	10	3.6673 ± 0.3872
TZAR101	Infected	14	4.283 ± 0.3954
TZAR101	Infected	18	4.1041 ± 0.468
TZAR101	Wounded	0	3.1224 ± 0.4132
TZAR101	Wounded	1	3.5559 ± 0.4189
TZAR101	Wounded	3	3.417 ± 0.5489
TZAR101	Wounded	4	3.1355 ± 0.4924
TZAR101	Wounded	6	3.1544 ± 0.4129
TZAR101	Wounded	10	3.2588 ± 0.5503
TZAR101	Wounded	14	4.4425 ± 0.4943
TZAR101	Wounded	18	4.1445 ± 0.6782

Table B.7 Relative expression levels of the *ZmWRKY67* gene (Tabular presentation of data found in Figure 4.7).

Variety	Treatment	DAI	Average RE ± Std. Error
B73	NTC	0	4.5741 ± 0.6268
B73	NTC	1	5.7112 ± 0.5749
B73	NTC	2	4.129 ± 0.8165
B73	NTC	3	4.7706 ± 0.9168
B73	NTC	4	4.6949 ± 0.7666
B73	NTC	6	4.8832 ± 0.5777
B73	NTC	10	4.98 ± 0.6245
B73	NTC	14	5.7672 ± 0.6002
B73	NTC	18	7.0945 ± 0.8116
B73	Infected	0	4.5741 ± 0.6268
B73	Infected	1	6.3025 ± 0.4661
B73	Infected	2	6.8137 ± 0.6722
B73	Infected	3	5.1759 ± 0.9168
B73	Infected	4	7.3331 ± 0.5477
B73	Infected	6	6.0848 ± 0.4745
B73	Infected	10	6.0976 ± 0.4665
B73	Infected	14	5.6676 ± 0.4916
B73	Infected	18	5.8053 ± 0.5746
B73	Wounded	0	4.5741 ± 0.6268
B73	Wounded	1	4.7322 ± 0.4661
B73	Wounded	2	5.1016 ± 0.6722
B73	Wounded	3	5.2648 ± 0.9168
B73	Wounded	4	5.4353 ± 0.535
B73	Wounded	6	5.1992 ± 0.4745
B73	Wounded	10	5.923 ± 0.5155
B73	Wounded	14	5.0197 ± 0.9838
TZAR101	NTC	0	4.1569 ± 0.4649
TZAR101	NTC	1	4.1229 ± 0.4917
TZAR101	NTC	2	4.3681 ± 0.9154
TZAR101	NTC	3	4.1477 ± 0.666
TZAR101	NTC	4	5.6183 ± 0.7907
TZAR101	NTC	6	5.5078 ± 0.5142
TZAR101	NTC	10	6.186 ± 0.4993
TZAR101	NTC	14	5.8948 ± 0.5027
TZAR101	NTC	18	6.6692 ± 0.5438
TZAR101	Infected	0	4.1569 ± 0.4649
TZAR101	Infected	1	5.6744 ± 0.42
TZAR101	Infected	2	5.9055 ± 1.0699
TZAR101	Infected	3	5.378 ± 0.666
TZAR101	Infected	4	6.1492 ± 0.5324
TZAR101	Infected	6	5.2516 ± 0.4199
TZAR101	Infected	10	6.0901 ± 0.4206
TZAR101	Infected	14	7.107 ± 0.4304
TZAR101	Infected	18	6.987 ± 0.5442
TZAR101	Wounded	0	4.1569 ± 0.4649
TZAR101	Wounded	1	4.6559 ± 0.46
TZAR101	Wounded	3	4.6637 ± 0.666
TZAR101	Wounded	4	5.1384 ± 0.577
TZAR101	Wounded	6	5.0922 ± 0.4535
TZAR101	Wounded	10	5.5064 ± 0.6263
TZAR101	Wounded	14	5.6901 ± 0.5603
TZAR101	Wounded	18	6.4557 ± 0.8674

Table B.8 Relative expression levels of the *ZmPR-1* gene (Tabular presentation of data found in Figure 4.8).

Variety	Treatment	DAI	Average RE ± Std. Error
B73	NTC	0	5.8688 ± 0.2979
B73	NTC	1	6.4165 ± 0.4086
B73	NTC	3	5.9306 ± 0.4606
B73	NTC	6	5.4143 ± 0.4116
B73	NTC	10	4.4013 ± 0.5276
B73	NTC	14	4.2734 ± 0.4324
B73	Infected	0	5.8688 ± 0.2979
B73	Infected	1	4.9694 ± 0.3007
B73	Infected	3	4.946 ± 0.4606
B73	Infected	4	5.1933 ± 0.4606
B73	Infected	6	5.1084 ± 0.3159
B73	Infected	10	4.6459 ± 0.3033
B73	Infected	14	4.3853 ± 0.3473
B73	Infected	18	7.0384 ± 0.5592
B73	Wounded	0	5.8688 ± 0.2979
B73	Wounded	1	5.1527 ± 0.3007
B73	Wounded	3	4.9854 ± 0.4664
B73	Wounded	4	5.0533 ± 0.4227
B73	Wounded	6	4.9262 ± 0.3159
B73	Wounded	10	4.8751 ± 0.4217
B73	Wounded	14	4.3593 ± 0.5362
TZAR101	NTC	0	4.5215 ± 0.2095
TZAR101	NTC	1	4.5672 ± 0.2902
TZAR101	NTC	3	4.8458 ± 0.3261
TZAR101	NTC	6	4.8557 ± 0.2901
TZAR101	NTC	10	4.6424 ± 0.3
TZAR101	NTC	14	4.2867 ± 0.3003
TZAR101	NTC	18	4.1691 ± 0.3261
TZAR101	Infected	0	4.5215 ± 0.2095
TZAR101	Infected	1	4.6815 ± 0.2127
TZAR101	Infected	3	4.8892 ± 0.3281
TZAR101	Infected	4	5.0258 ± 0.3129
TZAR101	Infected	6	5.0573 ± 0.2127
TZAR101	Infected	10	4.5159 ± 0.2131
TZAR101	Infected	14	4.8832 ± 0.2234
TZAR101	Infected	18	4.549 ± 0.3261
TZAR101	Wounded	0	4.5215 ± 0.2095
TZAR101	Wounded	1	4.6129 ± 0.2258
TZAR101	Wounded	3	4.5011 ± 0.3261
TZAR101	Wounded	4	4.8884 ± 0.2993
TZAR101	Wounded	6	4.6092 ± 0.2204
TZAR101	Wounded	10	4.2809 ± 0.3279
TZAR101	Wounded	14	3.7831 ± 0.2844
TZAR101	Wounded	18	4.7306 ± 0.4332

Table B.9 Relative expression levels of the *ZmNPR1* gene (Tabular presentation of data found in Figure 4.9).

Variety	Treatment	DAI	Average RE ± Std. Error
B73	NTC	0	6.0584 ± 0.2824
B73	NTC	1	6.5945 ± 0.375
B73	NTC	3	6.5668 ± 0.4262
B73	NTC	6	5.8436 ± 0.3778
B73	NTC	10	5.2878 ± 0.4774
B73	NTC	14	5.7506 ± 0.3995
B73	Infected	0	6.0584 ± 0.2824
B73	Infected	1	5.4462 ± 0.2839
B73	Infected	3	5.4847 ± 0.4262
B73	Infected	4	5.8725 ± 0.4262
B73	Infected	6	5.7178 ± 0.2965
B73	Infected	10	5.4938 ± 0.2846
B73	Infected	14	5.6825 ± 0.3232
B73	Infected	18	6.829 ± 0.51
B73	Wounded	0	6.0584 ± 0.2824
B73	Wounded	1	5.6083 ± 0.2839
B73	Wounded	3	5.7014 ± 0.4262
B73	Wounded	4	5.7662 ± 0.3944
B73	Wounded	6	5.2284 ± 0.2965
B73	Wounded	10	5.9126 ± 0.3886
B73	Wounded	14	5.3407 ± 0.4863
TZAR101	NTC	0	6.5175 ± 0.1998
TZAR101	NTC	1	6.4928 ± 0.2664
TZAR101	NTC	3	6.5431 ± 0.3014
TZAR101	NTC	6	6.1594 ± 0.2663
TZAR101	NTC	10	6.3347 ± 0.2734
TZAR101	NTC	14	6.6816 ± 0.2765
TZAR101	NTC	18	6.6981 ± 0.3014
TZAR101	Infected	0	6.5175 ± 0.1998
TZAR101	Infected	1	6.8313 ± 0.201
TZAR101	Infected	3	6.7246 ± 0.302
TZAR101	Infected	4	7.2242 ± 0.2893
TZAR101	Infected	6	7.0299 ± 0.2015
TZAR101	Infected	10	7.0257 ± 0.2013
TZAR101	Infected	14	7.1697 ± 0.2099
TZAR101	Infected	18	7.0968 ± 0.3014
TZAR101	Wounded	0	6.5175 ± 0.1998
TZAR101	Wounded	1	6.9024 ± 0.2121
TZAR101	Wounded	3	6.7681 ± 0.3014
TZAR101	Wounded	4	6.8637 ± 0.2789
TZAR101	Wounded	6	6.4655 ± 0.2069
TZAR101	Wounded	10	6.6437 ± 0.2991
TZAR101	Wounded	14	6.0666 ± 0.2627
TZAR101	Wounded	18	6.4509 ± 0.3975

Table B.10 Relative expression levels of the *ZmERF1* gene (Tabular presentation of data found in Figure 4.10).

Variety	Treatment	DAI	Average RE ± Std. Error
B73	NTC	0	4.3055 ± 0.8682
B73	NTC	1	4.4044 ± 0.9142
B73	NTC	3	3.9553 ± 0.9258
B73	NTC	6	3.4253 ± 0.9154
B73	NTC	10	2.5569 ± 0.9758
B73	NTC	14	3.7904 ± 0.9215
B73	Infected	0	4.3055 ± 0.8682
B73	Infected	1	3.3784 ± 0.8688
B73	Infected	3	2.7046 ± 0.9258
B73	Infected	4	3.7813 ± 0.9258
B73	Infected	6	3.258 ± 0.8747
B73	Infected	10	2.879 ± 0.869
B73	Infected	14	3.6386 ± 0.8868
B73	Infected	18	4.8315 ± 0.9827
B73	Wounded	0	4.3055 ± 0.8682
B73	Wounded	1	3.5866 ± 0.8688
B73	Wounded	3	3.7697 ± 0.9258
B73	Wounded	4	3.5666 ± 0.9061
B73	Wounded	6	3.0519 ± 0.8747
B73	Wounded	10	3.6043 ± 0.9185
B73	Wounded	14	4.0822 ± 0.9786
TZAR101	NTC	0	4.2514 ± 0.8169
TZAR101	NTC	1	4.129 ± 0.8419
TZAR101	NTC	3	4.2088 ± 0.8478
TZAR101	NTC	6	4.6031 ± 0.8419
TZAR101	NTC	10	4.2869 ± 0.8439
TZAR101	NTC	14	4.617 ± 0.8444
TZAR101	NTC	18	4.849 ± 0.8478
TZAR101	Infected	0	4.2514 ± 0.8169
TZAR101	Infected	1	4.3453 ± 0.8172
TZAR101	Infected	3	4.5187 ± 0.8478
TZAR101	Infected	4	4.8219 ± 0.8417
TZAR101	Infected	6	4.6597 ± 0.8173
TZAR101	Infected	10	4.6186 ± 0.8173
TZAR101	Infected	14	5.4602 ± 0.8204
TZAR101	Infected	18	5.3733 ± 0.8478
TZAR101	Wounded	0	4.2514 ± 0.8169
TZAR101	Wounded	1	4.3566 ± 0.8209
TZAR101	Wounded	3	4.2447 ± 0.8478
TZAR101	Wounded	4	3.8761 ± 0.837
TZAR101	Wounded	6	3.9377 ± 0.8192
TZAR101	Wounded	10	4.1502 ± 0.8561
TZAR101	Wounded	14	3.8792 ± 0.8384
TZAR101	Wounded	18	4.8136 ± 0.8772

APPENDIX C
GENOMIC LOCI HOMOLOGOUS TO THE MAIZE WRKY53.1 TRANSCRIPTION FACTOR IN B73

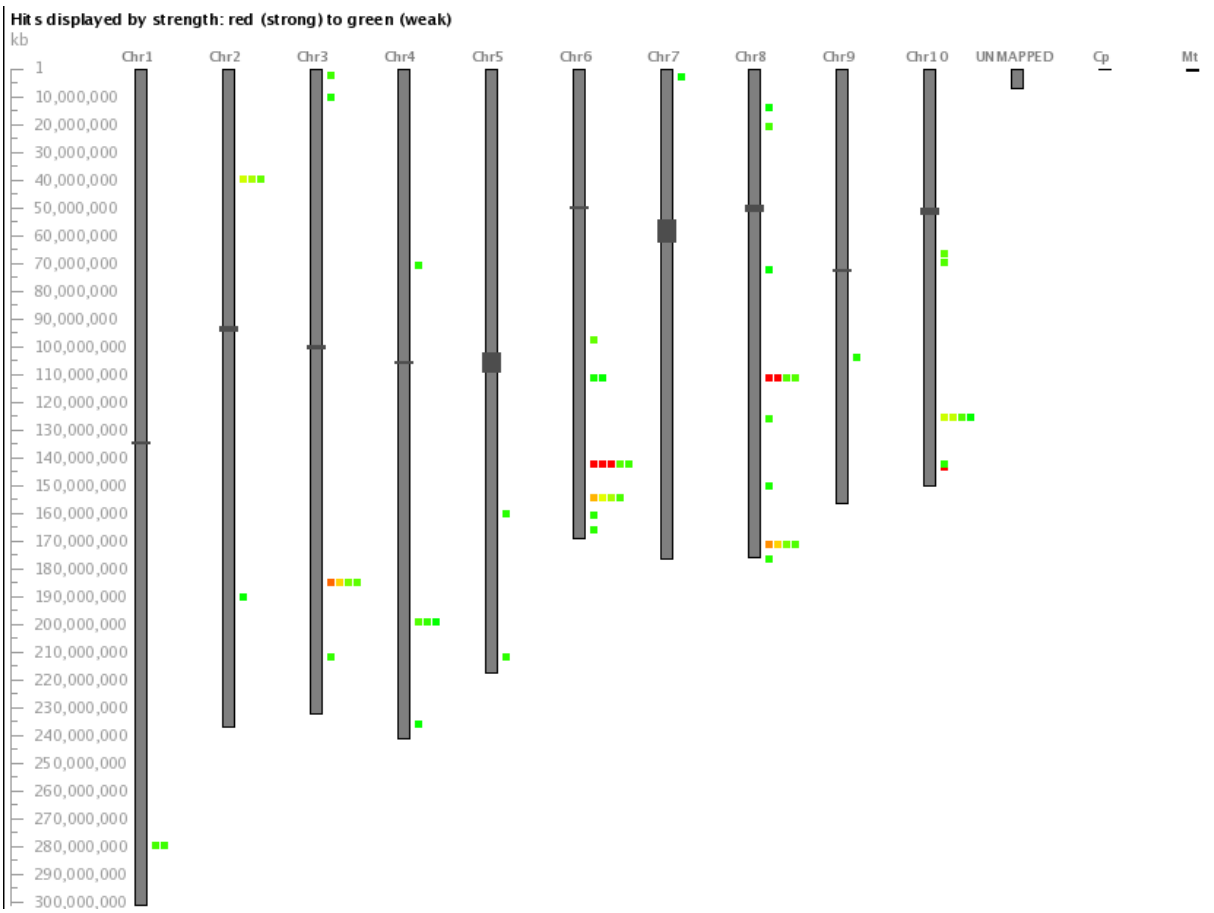

Figure C.1 Graphical representation of loci homologous to *ZmWRKY53.1* in the B73 genome. This graphic represents the whole genome view of homologous regions to *ZmWRKY53* in the B73 genome and was generated using the BLAST function for the Maize Genome Database (http:// http://blast.maizegdb.org/). The relative locations of homologous regions of the B73 genome to the nucleotide sequence of the *ZmWRKY53* gene are displayed by colored boxes. Red boxes represent regions highly similar (e-value < 1.0×10^{-100}) to the *ZmWRKY53* coding sequence. Yellow/orange and green boxes represent regions with moderate (e-value < 1.0×10^{-40}) and low homology (e-value > 1.0×10^{-40}), respectively.

APPENDIX D
SAS PROGRAMMING FOR STATISTICAL ANALYSIS OF REAL-TIME PCR DATA

Where the dataset labeled “one” is an imported spreadsheet containing the qPCR results and corresponding labels, and DV indicates the Dependent Variable, the gene examined in the analysis…

```
dm'log;clear;output;clear';
OPTIONS nodate nocenter pageno=1 ls=78 ps=55;

/*
Proc Print data=one;
Run;
Proc Mixed data=one;
Class Yr Var Plot Tnt DAI Plant;
Model DV2 = Var|Tnt|DAI / ddfm=kr;
Random Yr Plot(Yr Var) DAI*Plot(Yr Var) Plant(Yr Var Plot Tnt DAI);
Lsmeans Var|Tnt|DAI;
Run; Quit;
*/

%include "C:\Users\jfount4\Desktop\pmmix macro.sas";
%include "C:\Users\jfount4\Desktop\pdmix900.sas";

*PDMIX900 outm = means outd = diffs;

%PMMIX9v2(%nrbquote(Proc Mixed Data = one;
                    Classes Yr Var Plot Tnt DAI Plant;
                    Model DV2 = Var|Tnt|DAI / E3 ddfm = KR;
                    Random Yr Plot(Yr Var) DAI*Plot(Yr Var) Plant(Yr Var Plot Tnt DAI);
                    PMMTEST Var|Tnt|DAI / E BYLEVEL OM = OMDATA;
                    PMMOPTIONS NOEALL;));
```

VITA

Jake Clayton Fountain, a native of Ashburn, Georgia, was born in 1987 to Larry Fountain and Penny Rodgers. He graduated from Turner County High School in 2005. In 2007, he earned an Associate of Science degree, majoring in pre-professional medicine, at Abraham Baldwin Agricultural College in Tifton, Georgia. In 2010, he earned a Bachelor of Science degree, majoring in biology, from Georgia Southwestern State University in Americus, Georgia. Jake began his studies at Louisiana State University in the Department of Plant Pathology and Crop Physiology, in the fall of 2010, in the laboratory of Dr. Zhi-Yuan Chen. After obtaining his masters degree from LSU, Jake will be continuing his doctoral studies in the Department of Plant Pathology at the University of Georgia.