EXPRESSION PROFILING AND ANALYSIS OF RESISTANCE TO *USTILAGO MAYDIS* IN MAIZE AND TEOSINTE

by

SUCHITRA ARUN CHAVAN

(Under the Direction of Shavannor M. Smith)

ABSTRACT

Ustilago maydis (*U. maydis*) is the causal agent of corn smut and is responsible for significant yield losses of approximately $1.24 billion annually in the United States. The infected plants show gall formation on all the aerial parts of the plant. Significant economic loss occurs due to infected kernels that cannot be used for food or fuel production. Several methods are utilized to control corn smut disease however; host resistance is the only practical method for managing common smut. Currently, there are no known maize lines that are resistant to *U. maydis*. It is therefore necessary to identify new sources of resistance to *U. maydis*. We have identified maize, teosinte and maize-teosinte introgression lines (NILs) with a high level of resistance to *U. maydis*. This is the first report of the identification of new sources of resistance to *U. maydis* from teosinte and maize-teosinte NILs. The teosinte introgressed region present in the resistant NIL is 3.6Mbp in size and carries 7 genes that may be contributing to the resistant phenotype. To identify genes expressed in response and *U. maydis* infection in an incompatible reaction, transcriptome profiling was conducted on maize genotypes demonstrating resistance

and susceptibility to *U. maydis.* Among the 17,555 genes monitored using Affymetrix GeneChip maize genome array, 5,639 genes showed significant differential expression between the control and *U. maydis* inoculated maize lines at 24 hours post inoculation (hpi). The up-regulated genes (≥1.5 fold change) were grouped into 7 categories, and were classified as genes coding for proteins associated with defense related genes, enzyme families, receptor like kinases, photosynthesis, regulation overview and transcription. The down regulated genes (≤ 1.5 fold change) were grouped into 10 categories representing genes involved in enzyme families, hormones, plant glycolysis, photosynthesis, metabolism, cell function, transcription, defense related genes, receptor like kinases and regulation overview. These findings provide insight into the complexity of biotrophic interactions in an incompatible interaction and indicate that the activation of plant defenses in response to *U. maydis* infection is similar to other biotrophic interactions.

INDEX WORDS: Maize, teosinte, maize-teosinte introgression lines, *diploperennis, luxurians, parviglumis,* NILs, *Ustilago maydis,* disease rating scale, host-pathgoge interactions, biotrophic pathogen, disease resistance, incompatible interaction, phenotypic reaction, days post inoculation (dpi), hours post inoculation (hpi)

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DEDICATION

I would like to dedicate this dissertation to my parents (Mr. Arun Kumar Chavan and Mrs.Nanda Arun Chavan) whose love, support, motivation and inspiration have helped me throughout my life. My countless thankfulness and love goes to my life partner Dr. Satyanarayana Lagishetty whose love, moral support, and encouragement made everything possible. I would like to thank my daughter Siddhi Lagishetty and brother Amit Chavan for their love and support.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Purpose and significance of the study

Host resistance is the only practical method for managing common smut. The overall goal of this project is to identify new sources of resistance to *Ustilago maydis* (*U. maydis)* and to study the differentially expressed genes in maize in response to *U. maydis* infection. In order to accomplish our goal we will identify genes involved in plant defenses in maize and teosinte in response to *U. maydis* infection, and determine their potential function thereby facilitating a better understanding of the molecular and genetic processes of disease resistance. This will be done utilizing microarray analysis. Maize lines with "best" resistance to *U. maydis* and susceptible lines will be inoculated with the wild type *U. maydis* strain. RNA extracted from the experimental types at different time points will be used to probe microarrays. Comparison of the expression pattern of plant defense genes in the "best resistant" and susceptible maize lines will allow us to identify genes that are differentially regulated, select candidate genes involved in plant defenses and determine their potential function.

Teosinte like its derivative maize is also infected by corn smut (*U. maydis*). The morphological and genetic similarity of teosinte and maize in addition to the similar phenotypic response to corn smut indicate the two species may share key components in plant defenses and the activation of defense genes. The domestication of maize over the years may have led to significant changes in its plant defense gene expression. As a result,

analyzing the phenotypic response of teosinte on *U. maydis* inoculation will enable us to identify new sources of resistance from the wild progenitor. This work represents the first report of new potential sources of resistance to *U. maydis* from the wild progenitor teosinte. The differentially expressed resistance and plant defense genes seen in resistant maize plants can be employed in maize breeding programs to improve resistance of commercially grown maize plants against *U. maydis*.

The analysis of maize-teosinte introgression lines (NILs) that confer resistance to *U. maydis* and will enable us to identify the teosinte introgressed region responsible for resistance. Identification of the introgressed regions will facilitate the identification and characterization of the resistance genes in the introgressed region specifically controlling resistance. This knowledge will provide a better understanding of the mechanism of resistance to *U. maydis*. This information is crucial for the design of novel strategies to develop maize cultivars with high levels of resistance to *U. maydis*. This work will have major implications for the characterization of resistance mechanisms that may be more durable and the development of novel strategies to control *U. maydis*.

The overall goal of this work was to identify and characterize differentially expressed plant defense genes in *Zea mays* in response to *U. maydis* and compare these data in maize and teosinte lines with the aim of identifying new sources of resistance from the wild progenitor teosinte.

Literature review

Plant-Pathogen Interactions

Plants and pathogens co-exist in nature. However, many plant pathogens attack the plants in order to obtain nutrition, survive and reproduce. The major classes of plant

pathogens include fungi, oomycetes, phytoplasma, virioids, bacteria, viruses, nematodes and higher plants. Plants have evolved many different defense mechanisms to defend themselves against pathogen attack.

Preformed structural barriers are one of the defense mechanisms and are considered to be the first lines of plant defenses.Preformed barriers include trichomes, thick cuticle layer, fatty acids and lignin deposition in the cell wall (Glazebrook *et al.*, 2005). In addition, to preformed structural barriers plants produce antimicrobial compounds such as phytoanticipins, tannins and phytoalexins (Vanoosthuyse *et al.*, 2001). The aforementioned plant defense mechanisms are typically more than adequate to fend off potentially invading plant pathogens. However, some of the more aggressive plant pathogens are able to overcome the preformed structural barriers. In response to this, plants have evolved a second layer of defense that is more elaborate and results in the activation of many biochemical processes inside the plant cell (Durrant 2004 and Truman 2007). For example, if the pathogen is capable of overcoming preformed barriers in a resistant plant, the pathogen effector proteins (*Avr* gene product) are recognized by plant resistance gene (R-genes) proteins. Interactions (directly or indirectly) of the *Avr* and R-gene protein products result in activation of defense responses. This facilitates cell wall lignification, production of reactive oxygen species (ROS), activation of pathogenesis related (PR) genes, hypersensitive response (HR) at the site of infection and production of secondary metabolites like tannins, phytoalexins and phenolic compounds (Van Loon *et al.*,2006). Conversely, in a susceptible reaction the plant does not recognize the *Avr* gene product due to the lack of the cognate R gene. This causes the pathogen to grow and reproduce in the plant resulting in disease and death.

Resistance Gene Classes and Structure

R-genes have been extensively studied in many plant species with respect to structure, function and evoloution. To date, seventy R-genes have been cloned and characterized (Liu *et al.*, 2007). Based on their structure, the majority of the characterized R-genes are grouped in to four major classes; (1) nucleotide binding site-leucine rich repeat (NBS-LRR), (2) extracellular LRR (NBS-TM-LRR), (3) protein kinase and (4) receptor kinase. The NBS-LRR class is considered the largest classes of R-genes because the majority of the cloned R-genes are of this type (Goff 2002). It has been shown that the NBS domain contains several highly conserved motifs that function in the activation of plant defenses. These include P-loop, kinase 1a, kinase 2 (also known as Walker's A and B boxes respectively), kinase 3 as well as several blocks of conserved motifs with unknown function (RNBS-A, RNBS-C, GLPL, RNBS-D and MHD) (DeYoung *et al.*, 2006). The MHD motif is highly conserved in plant NBS-LRR proteins (DeYoung *et al.*, 2006). The structure of NBS domain of the *Caenorhabditis elegans* cell death protein CED-4 is similar to mammalian apoplastic protease activating factor 1 (Apaf-1) and is composed of four subdomains; a three-layered α-β domain (constituting the P- loop) followed by a helical domain, and two tandem extended winged helix domains (ARC1and ARC2) (DeYoung *et al.*, 2006).

Activation of plant defenses is highly regulated. One of the principle regulation processes in plant defenses involves the conformational change of the R-gene protein. NBS-LRR proteins are considered to be active or inactive based on their conformation. For example, in the inactive state, ADP is bound to the NBS domain. However, the presence of the pathogen effector protein alters the structure of the NBS-LRR protein allowing the exchange of ADP for ATP resulting in the active state of the NBS-LRR protein. Binding of

ATP to the NBS domain causes activation of the downstream signaling molecules resulting in the activation of plant defenses. Conversely, dissociation of the pathogen effectors and modified effector targets along with the hydrolysis of ATP returns the NBS-LRR protein to its inactive state (DeYound *et al.*, 2006). The N-terminal end of the NBS-LRR protein mediates the physical interation between resistance proteins and pathogen effector targets, for the resistance proteins that use an indirect recognition mechanism. This was first reported for the *RPM1-RIN4* interaction in *Arabidopsis thaliana*. *RPM1* confers resistance to the bacterial pathogen *Pseudomonas syringae*. This interaction leads to the phosphorylation of *RIN4* which is detected by the NBS-LRR protein *RPM1*, resulting in activation of plant defenses (DeYoung *et al.* 2006). Thus, the N-terminal end of plant NBS-LRR proteins may be involved in both detection of the pathogen signal and activation of downstream signaling molecules resulting in resistance (DeYound *et al.*, 2006).

The leucine rich repeat domain is located at the carboxy-terminal (C-terminal) end of the plant NBS-LRR proteins and is characterized by the presence of an xxLxLxx repeat sequence, where 'x' represents any aliphatic amino acid and 'L' is the amino acid leucine. The structure of the repeat motif consists of a β-strand and α-helix, where the β-strands form a parallel β-sheet, in which adjacent strands are more closely packed than the opposed helices, resulting in the characteristic curved structure (Kobe and Deisenhofer 1994). The β-sheet region has been shown to form a protein interaction surface and therefore contains most of the sequence variation as a result of diversifying selection in this region (DeYoung *et al.*, 2006). The exterior of the protein is composed of aliphatic amino acids. The aliphatic amino acids interact with solvent molecules, while the hydrophobic leucine amino acids are buried inside the protein. The C-terminal end of the LRR domain interacts directly or indirectly with

the pathogen effector molecules and controls recognition specificity of the pathogen effector molecules (DeYoung *et al.*, 2006). This was demonstrated at the *L* locus in flax with domain swap experiments between R-genes with different recognition specificities (Dodds *et al.*, 2006). The *L* locus has thirteen alleles (*L*, *L1* to *L11*, and *LH*) that confer different race specificities against the flax rust fungus *Melamspora lini.* Substitution of the *L6* and *L10* LRR domain with the *L2* LRR domain resulted in the *L2* race specificity for both *L6* and *L10* indicating that the LRR region is responsible for pathogen recognition and controls pathogen specificity (DeYoung *et al.*, 2006).The NBS-LRR class is further divided into two distinct categories based on their N-terminal modifications. The Toll-interlukin NBS-LRR (TIR-NBS-LRR) category is characterized by an N-terminal TIR domain. The TIR domain is similar to the intracellular signaling domain of the *Drosophila* Toll protein and mammalian interleukin-1 receptor protein. The TIR and NBS domains are highly conserved, while the TIR domain has been observed only in dicots. Both domains function in triggering innate immunity in response to recognition of pathogen effectors. However, the TIR domain has also been associated with resistance specificity. For example, non-functional resistance genes were seen with recombinants at the flax *L* locus combining TIR and NBS domains indicating that TIR domain can alter recognition specificity (Rafiqi *et al.*, 2009). To elaborate replacement of the TIR-encoding region of the *L6* allele with the corresponding regions of *L2* or *LH* by recombination changed the specificity of the allele from *L6* to *L7*. Therefore, intramolecular interactions between the TIR, NBS and LRR domains are essential for recognition of the pathogen and activation of plant defenses at the *L* locus. Examples of the TIR-NBS-LRR resistance gene class are the tobacco *N* locus and the flax *L6* locus, that

confers resistance to tobacco mosaic virus (TMV) and *Melampsora lini*, respectively (Mestre and Baulcombe 2006).

The second category of the NBS-LRR class is the coiled-coiled nucleotide binding site leucine rich repeat (CC-NBS-LRR) protein, which is characterized by the presence of two or more alpha helices that form a supercoil and contain a heptad sequence (abcdefg), where hydrophobic amino acids are 'a' and 'd' and hydrophilic amino acids are 'e' and 'g'(Nooren *et al.*, 1999). The coiled-coiled (CC) domain is responsible for activating the downstream signaling cascade resulting in activation of plant defenses (Meyers *et al.*, 2003). The *Arabidopsis RPM1* resistance gene is the best characterized example of the coiled-coiled NBS-LRR class. *RPM1* confers resistance to *Pseudomonas syringae* pv. *glycinea* and *Pseudomonas* pv. *maculicola*, by detecting the phosphorylation of *RPM1*-interacting protein 4 (*RIN4*) due to the presences of *AvrB* and *AvrRpm1* pathogen effectors (Mackey *et al.*, 2002). Based on the structure of the NBS-LRR resistance genes, it is predicated that they are located inside the plant cell and interact with effector proteins in the cytoplasm.

The second class of R-genes has an NBS domain, a transmembrane (TM) domain and an extracellular LRR. As indicated by the name, this class differs from the first class in that it is characterized by the presence of a TM domain and an extracellular LRR domain. The extracellular LRR domain functions in recognition of the pathogen effector molecules outside the plant cell. The transmembrane domain anchors the protein to the membrane and connects the external LRR region with the internal NBS region of the protein. After the effector molecule binds (directly or indirectly) to the extracellular LRR domain, the transmembrane domain transmits the recognition signal to other signal transduction proteins within the cell in order to activate plant defenses. The tomato *Cf* genes (*Cf*-4 and *Cf*-9) are

the best characterized examples of the NBS-TM-LRR class of resistance genes. There are thirteen *Cf* genes in tomato that confer race-specific resistance to *Cladosporium fulvum* (*Cf*). The amino acid sequence of the *Cf*9 gene suggests that there are twenty-eight LRRs, most of which are extracellular, indicating that this gene interacts with the corresponding effector molecules outside the plant cell. The N-terminus of the *Cf*-9 gene has a signal peptide sequence responsible for transport across the membrane. The C-terminus contains a transmembrane domain with a short 28 amino acid tail that is likely cytoplasmic. The extracellular LRR domain interacts with the *Avr9* pathogen effector from *Cladosporium fulvum*. As a result of this interaction, a signal is transmitted through the TM domain, to the inside of the plant cell activating plant defenses (Hammond-Kosack and Jones 1997).

Protein kinases are the third class of R-genes. An example of this class is the *Pto* protein kinase in tomato. This class of R-gene does not have a transmembrane domain and is therefore predicted to be located inside the plant cell. *Pto* encodes a Serine/Threonine (Ser/Thr) protein kinase that requires the NBS-LRR protein *Prf* to interact with the pathogen effector *avrPto* from *Pseudomonas syringae* (Salmeron *et al.*, 1996). This interaction initiates a phosphorylation cascade which further phosphorylates another Ser/Thr protein kinase, *Pti1* (Scofield *et al.*, 1996). The mitogen-activated protein kinase (MAPK) cascade is then activated followed by the hypersensitive response (HR), a form of programmed cell death (PCD) in the host plant cells, resulting in host resistance (Liu *et al.*, 2007).

The fourth class of R-genes is the receptor kinase class, which is characterized by an extracellular LRR domain, a transmembrane domain (TM) and intracellular kinase (Song *et al.*, 1995). The effector molecules are recognized by the LRR domain and this recognition signal is transmitted through the TM domain to the intracellular kinase domain, which further

activates downstream signaling cascade resulting in activation of plant defense. The rice *Xa21* gene is the best characterized receptor kinase R-gene and confers resistance to *Xanthomonas oryzae pv oryzae* (*Xoo*). The extracellular leucine-rich repeat (LRR) domain, serves as the receptor for recognizing specific pathogen *Xoo* effectors outside the plant cell. The recognition signal is then transmitted to the internal kinase domain through the transmembrane domain resulting in the activation of downstream signaling cascades and plant defenses (White and Yang 2009).

Plant-Pathogen Recognition

Plants recognize the presence of pathogens either directly or indirectly. Direct pathogen recognition was first explained by the gene-for-gene model put forth by Flor in 1971 (Flor, 1971). This model is based on the genetic interaction of a dominant plant R-gene and pathogen avirulence (*avr*) gene. According to this model, the plant R-gene functions as a receptor for pathogen derived ligands/effectors encoded by *avr* genes. In an incompatible interaction, the plant R-gene recognizes the pathogen *avr* gene resulting in host plant resistance. Conversely, if the plant R-gene fails to recognize the pathogen *avr* genes because; (1) the plant does not carry the corresponding R-gene, (2) the pathogen does not carry the corresponding *avr* gene, or (3) both the R-gene and *avr* gene are absent, then the interaction is considered to be "compatible" resulting in host plant susceptibility (Flor, 1971). Therefore, the presence of both the R-gene and corresponding a*vr* gene is essential for overcoming pathogen infection. This type of interaction is described as the gene-for-gene model and can be either direct or indirect. For example, the *Pi-ta* R-gene in rice confers resistance to *Magnaporthe grisea*. (Jia *et al.*, 2000). By using the yeast-two-hybrid (Y2H) system and *in*

vitro binding assays, it was demonstrated that *AVR-Pita* directly binds the *Pi-ta* LRR domain. Mutations in the LRR region abolished the binding between *AVR-Pita* and the *Pi-ta* R-gene resulting in susceptibility and confirming the direct interaction (Rafiqi *et al.*, 2009). The *Pita* gene is one of only two examples of a resistance gene protein interacting directly with the corresponding *Avr* protein. (Jia *et al.*, 2000).

The ability of the plant to recognize the presence of a pathogen is essential in order to activate plant defenses. Activation of plant defenses is kept under strict genetic control. The initial response of the plant to the pathogen was described by Jones and Dangl 2006 (Jones and Dangl *et al.*,2006) with the four phased 'zig-zag model' and sheds light on the specificity of plant defense activation. According to phase 1 of the zig-zag model, plant cell surface-located transmembrane receptors, referred to as pathogen recognition receptors (PRRs), detect the conserved molecular signature of pathogens known as microbial/pathogen associated molecular patterns (MAMPS/PAMPS). Several PRRs have been identified and include receptor like kinases and receptor like proteins that are attached to the plant cell surface and resemble animal Toll-like receptors (TLRs) (Zipel *et.al.*, 2008). Examples of characterized PAMPs include lipopolysaccharides of Gram-negative bacteria, conserved epitope elf 18 from the bacterial translation factor EF-Tu, fungal-oomycete cellulose binding elicitor proteins and peptide motifs in bacterial flagella (Chisholm *et al.*, 2006). The perception of PAMPS by PRRs results in activation of the MAP kinase signaling cascade resulting in the activation of basal defense responses, known as PAMP triggered immunity (PTI) (Rafiqi *et al.*, 2009).

In phase 2 of the zig-zag model, successful pathogens are capable of overcoming PTI and deploying effectors that contribute to pathogen virulence, resulting in effector triggered

susceptibility (ETS) in the host plant. Natural selection drives the pathogen to acquire new effectors in order to survive. This is accomplished by shedding the recognized effectors or by acquiring additional effectors that suppress effector triggered immunity (ETI) in the host plant. However in phase 3, newly evolved NBS-LRR proteins recognize (directly or indirectly) the pathogen effectors activating effector triggered immunity (ETI) in the plant resulting in host plant resistance. Plants acquire new R-genes through various mechanisms including diversifying selection and recombination that are capable of recognizing the new pathogen effectors (Jones and Dangl 2006; Glowacki *et al*., 2011). Even though PTI and ETI use different receptors to recognize pathogen attack, the downstream signaling cascade for both of the responses are interconnected, this results in resistance (Panstruga et al., 2009; Truman et al., 2006).

Plant-Pathogen Interaction Molecular Models

The gene-for-gene model was the first model to describe the interaction between a plant R-gene protein and an *Avr* pathogen effector protein. However, the majority of the cloned and characterized R-gene proteins do not interact directly with the corresponding *Avr* effector proteins. Few R-genes are capable of recognizing numerous pathogen effectors, indicating that there are additional internal plant proteins that are involved in recognition of pathogen effectors. Therefore, additional models have been proposed that describe indirect R-gene protein and effector protein interactions. Based on the proposed models, the *Avr* protein typically interacts with an internal plant protein that causes a conformational change in the newly formed protein complex. This change is detected by the R-gene which in turn sends a signal to activate plant defenses. In order to explain this phenomenon the ‘guard

model' was proposed by Ven der Bienzen and Jones in 1998 (Ven der Bienzen and Jones 1998). According to this model, the pathogen *Avr* proteins interact with a specific target internal plant protein designated the 'guardee'. This interaction is monitored by the R-gene protein designated the 'guard' (Jones *et al.*, 2006). For example, the bacterial effectors *AvrRpm1* and *AvrB* are indirectly detected separately by the NBS-LRR R-gene protein *RPM1* (guard) in *Arabidopsis thaliana*. *AvrRpm1* and *AvrB* separately interact with another internal plant protein (guardee) *RIN4* (*RPM1* interaction protein 4). Both interactions lead to the phosphorylation of *RIN4* which causes a conformational change in the newly formed *Avr* gene-guardee protein complex (*AvrRpm1-RIN4* or *AvrB-RIN4)*. The conformational change is detected by *RPM1* resulting in activation of plant defenses (DeYoung *et al.*, 2006). Thus, multiple effectors like *avrRpm1* or *avrB* can be recognized by the same R-gene (*RPM1*) due to the presence of the specific target guardee protein (*RIN4*) (Mackey *et al.*, 2002). In recent years other models have been proposed for interactions that do not conform to the gene-for-gene or the guard model. However, more evidence is needed to support the newly proposed models (Van der Hoorn and Kamoun, 2008).

Down Stream Signaling

After recognition of the pathogen by the plant, several biochemical changes occur inside the plant cell that leads to the activation of plant defenses. One of the most immediate responses that occurs in an incompatible interaction is the movement of Ca^{2+} inside the plant cell and the movement of K^{+} and H^{+} outside the plant cell, which maintains an electrochemical balance in the cell (Scheel 1998). Additionally, the perception of microbe associated molecular patterns (MAMPs) by PRR triggers an increase in the intracellular Ca^{2+}

concentration. Movement of the three ions across the cell membrane initiates the production of reactive oxygen species (ROS) also known as the oxidative burst. The oxidative burst occurs in two phases and requires sustained Ca^{2+} influx (likely activates NADPH oxidases), indicating that the production of ROS occurs downstream of Ca^{2+} influx (Overmyer *et al.*, 2003). In Phase I of ROS production, the oxidative burst is rapid and short lived, does not cause cell death (hypersensitive response; HR) and occurs in both compatible and incompatible reactions,. Conversely, during Phase II, the oxidative burst lasts longer and occurs only in an incompatible interaction (Alvarez *et al.*, 1998). The prolonged production of ROS is harmful to the plant cells. Hence, superoxide on the outside of the cell membrane is rapidly converted to hydrogen peroxide (H_2O_2) by superoxide dismutase. However, a balanced production of ROS inside the plant cell is required to activate plant defenses. Salicylic acid (SA) biosynthesis is then induced in response to ROS accumulation inside the plant cell resulting in supplementary cell-wall strengthening as a first line of mechanical defense (Hückelhoven and Kogel, 2003). Ca^{2+} elevation also increases the amount of Ca^{2+} bound to calmodulin (CaM). The Ca^{2+}/CaM complex regulates the synthesis of downstream signaling components nitric oxide (NO) and H_2O_2, which are essential for the development of the hypersensitive response (HR). Nitric oxide (NO) is a small highly mobile molecule which interacts with hydrogen peroxide during HR induction (De Pinto *et al.*, 2002). Therefore, SA and NO induction are downstream of ROS production. This induction is described as the initiation step of programmed cell death (PCD). During the initial stages of programmed cell death, SA and ethylene (ET) suppress the production of jasmonic acid (JA) (Overmyer *et al.*, 2003). Suppression of JA is required for cell death to occur at the site of infection and restricts the growth of the pathogen to the infection site (HR). A burst of ET spreads to

adjacent cells and induces PCD in those cells and is described as the propagation step of PCD. ET is required for the continuous production and accumulation of ROS, which drives cell death (Overmyer *et al.*, 2003). To halt the spread of PCD. ROS is no longer produced. In the absence of ROS, JA accumulates in the neighboring cells. JA in turn suppresses SA, ET and PCD. This process is described as the containment step of PCD (Overmyer *et al.*, 2003).

Pathogenesis Related Proteins

Pathogenesis related (PR) proteins were first defined by Antoniw *et al.*, in 1980 as "proteins encoded by the host plant but induced only in pathological or related situations, including situations of non-pathogenic origin" (Antoniw *et al.*, 1980). To be classified as a PR protein, a protein must be newly expressed upon biotic and/or abiotic stress (Edreva, 2005). The protein should be expressed in all pathological conditions, not only the resistant or hypersensitive response condition but also during parasitic attack by nematodes, insects and herbivores (Edreva, 2005).

The first PR proteins were characterized in tobacco using molecular techniques. Five main groups of PR proteins were identified and designated PR-1 to PR-5, with the PR numbers in order of decreasing electrophoretic mobility (Bol *et al.*, 1990). The most abundant among these five groups was PR-1, reaching up to 1-2 % of total leaf proteins. Due to their significant amino acid sequence similarity with the sweet tasting protein in the fruits of the tropical plant *Thaumatococcus daniellii*, the PR proteins of group 5 were named as thaumatin-like (TL) proteins , which also included the osmotins (Singh *et al.*, 1987). In 1994 the nomenclature of PR proteins was modified based on their grouping into families sharing

amino acid sequences, serological relationships, and enzymatic or biological activity. This resulted in the identification of eleven PR families (PR-1 to PR-11) in tobacco and tomato. Among these 11 families, PR-8 and PR-10 were present in cucumber and parsley, respectively. Further, the study of PR genes led to the identification of three novel families (PR-12, PR-13 and PR-14), present in *Arabidopsis* and barley (Van Loon and Van Strien, 1999). In 2004 Park *et al.*, identified two new PR proteins namely germins (PR-15) and germin like protein (GLPs, PR-16). Thus, it was concluded that in order to be included in the category of PR proteins, a plant protein should be induced by a pathogen in tissues that do not normally express it and the expression should be seen in at least two different plant-pathogen combinations, or if the expression is seen in a single plant-pathogen combination it must be confirmed in different laboratories (Van Loon and Van Strien, 1999: Edreva, 2005).

PR proteins are currently grouped into 17 families, PR-1 through PR-17 (Van Loon *et al.*, 1999, Christensen *et al.*, 2002). The PR-1 family consists of proteins that have antifungal activity. PR-2 family comprises of β-1, 3 glucanases which hydrolyze β-1, 3 glucan present in the cell wall of fungi. The PR-3 family consists of chitinases which cleave the cell wall chitin polymers *in situ*, resulting in a weakened cell wall and rendering the fungal cells osmotically sensitive. The PR-4, PR-8 and PR-11 families consist of chitinases belonging to various chitinase classes (I-VII). The PR-5 family consists of thaumatin like proteins. Other PR families include proteinase inhibitors (PR-6), endoproteinase (PR-7), peroxidases (PR-9), ribosome inactivating proteins (PR-10), defensins (PR-12), Thionins (PR-13), lipid transfer proteins (PR-14), oxalate (oxidases /germins) (PR-15) and oxalate like proteins (oxidases-like proteins /germin like protein) (GLPs, PR-16) (Sels *et al.*, 2008).

PR proteins are present in the primary and secondary cell walls of infected plants; they are also seen in cell wall appositions (papillae) deposited at the inner side of cell wall in response to fungal attack (Jeun, 2000). PR proteins are not only present in plants but they are also seen in cell walls of invading fungal pathogens and in the space formed between cell walls and invaginated plasma membrane of fungi (Jeun, 2000; Jeun and Buchenauer, 2001). Pathogen-derived elicitors such as glucan, chitin, fungus secreted glycoproteins, peptides, proteins of elicitin family and protein products of avirulence genes are major inducers of PR proteins (Kombrink *et al.*, 2001; Edreva *et al.*, 2002, Hennin *et al.*, 2001). Apart from the above mentioned inducers, chemicals such as salicylic acid (SA), polyacrylic compounds, fatty acids and inorganic salts are also associated with PR protein induction (Hennin *et al.*, 2001). Physical stimuli such as wounding, UV-B radiation, osmotic shock, extreme temperature conditions and severe variation in water content, are effective PR protein inducers. In addition to these, plant hormones like ethylene (ET), jasmonates (JA), abscisic acid (AA), kinetin, auxins also induce PR proteins (Edreva, 1990, 1991; Van Loon, 1999; Fujibe *et al.*, 2000). Previously it was considered that PR proteins were induced by environmental and developmental stimuli, however recent reports (Edreva, 2005) indicate that they are constitutively present in different plant organs and seeds even in absence of stress condition, indicating that they have potential function as preformed defense barrier (Edreva, 2005).

The characteristic function of PR proteins is their proteinase-inhibitory, membrane permeability and hydrolytic activity that is responsible for their antifungal, antibacterial, nematicidal, antiviral and insecticidal properties (Van Loon and Van Strien, 1999; Van Loon, 2001; Selitrennikoff, 2001). The PR proteins that are hydrolytic enzymes (β-1, 3-glucanases,

chitinases and proteinases) act on chitin, glucan and fungal cell wall proteins (Van Loon and Van Strien, 1999; Van Loon, 2001; Selitrennikoff, 2001). However, PR-18 has lysozyme activity and is capable of disrupting gram negative bacteria (Van Loon and Van Strien, 1999; Van Loon, 2001; Selitrennikoff, 2001). In 1987 Legrand's (Legrand *et al.,* 1987) research group identified chitinase activity associated with four members of group 3 PR proteins and β-1, 3-glucanase activity associated with four members of group 2 PR proteins (Legrand *et al.*, 1987). The chitinase activity of PR proteins was not restricted to group 3 PR proteins and was also identified in PR-4, PR-8 and PR-11. Further studies with PR proteins showed that PR-7, PR-9, PR-10 and PR-8 had proteinase, peroxidase, ribonuclease and lysozyme activities. Recent studies identified new PR proteins including defensins (PR-12), thiols (PR-13), lipid-transfer proteins (LTPs) (PR-14) and thaumatin-like proteins (PR-5), all having membrane permeabilizing function (Van Loon and Van Strien, 1999, Van Loon, 2001; Selitrennikoff, 2001; Park *et al.*, 2004). The enzymatic activity of PR proteins allows them to damage or break down the pathogen and release chitin and glucan fragments from fungal cell walls which serve as elicitors and induce downstream signaling cascades resulting in activation of plant defenses (Lawrence *et al.*, 2000; Kombrink *et al.*, 2001).

It has been shown that the PR proteins constitutively expressed in seeds and plant organs have high fungitoxicity of seed osmotins and thaumatin-like proteins validating the defense function of PR genes (Vigers *et al.*, 1992; Abad *et al.*, 1996). For example, in tobacco group 5 PR proteins were characterized as osmotins and inhibited the growth of *Candida albicans, Neurospora crassa* and *Trichoderma reesei*. Constitutive expression of group 5 PR protein osmotins caused the hyphal tips of *N. crassa* to burst rapidly, suggesting that tobacco thaumatin-like PR proteins are antifungal by a membrane permeabilization

mechanism (Abad *et al.*, 1996). Apart from constitutive expression, some PR proteins show cell, tissue, organ and development-specific expression suggesting that PR proteins have functions beyond plant defenses. This was demonstrated in tobacco seed germination, where glucanse activity of PR-2d weakened the endosperm allowing the radicle to protrude (Benhamou, 1991; Jeun and Buchenauer, 2001). In another study, PR-3 and PR-4 chitinase homologous showed morphogenetic factors in carrot embryogenesis (Kragh *et al.*, 1996) and PR-5 (osmotins) was highly induced in tomato and tobacco plants with high osmotic stress suggesting that these PR genes are involved in embryogenesis and osmotic adaption (Hanfrey *et al.*, 1996).

The role of PR proteins in plant defenses has been well documented in many plant pathogen interactions by analyzing increased expression of PR proteins in inoculated resistant plants in comparison to inoculated susceptible plants. Differential expression of PR protein β-1, 3-glucanase was observed between two potato cultivars with different degrees of resistance to *Phytophthora infestans* (Tónon *et al.*, 2002). A four-fold increase in expression of β-1, 3-glucanase was observed for the resistant cultivar (Pampeana INT) with respect to healthy tubers 14 hours after inoculation. However, the susceptible cultivar (Bintje) showed lower expression of β-1, 3-glucanase as compared to resistant cultivar, indicating that the expression of β-1, 3-glucanase is due to inoculation with *Phytophthora infestans*. Additionally, injection of purified race-specific elicitors, *AVR4* and *AVR9* in to tomato *Cf4* and *Cf9* genotypes, induced differential expression of two PR proteins acidic chitinase and acidic 1,3-beta-glucanase. This was observed most abundantly in resistant genotypes thus confirming that PR genes show increased expression in, incompatible interaction as compared to compatible *C. fulvum*-tomato interactions (Wubben *et al.*, 1996).

Plants with high levels of natural resistance show constitutive expression of PR proteins. One of the best characterized examples of PR protein constitutive expression was reported for an apple cultivar (*Malus domestica* cv. Elstar) resistant to apple scab (Gau *et al.*, 2004). Protein analysis of apple leaves collected from the resistant apple cultivar that were infected with apple scab expressed three PR proteins, β-1, 3-glucanase, chitinase thaumatin like proteins and cysteine-like protease. These same proteins were expressed at a lower level in an uninoculated apple cultivar (*M. domestica*cv Remo) that was resistant to apple scab, powdery mildew and fire blight, indicating constitutive production of PR proteins in the resistant cultivar. Supporting these findings, constitutive expression of PR proteins in transgenic plants over expressing PR genes is accompanied by increased plant resistance to various pathogens. This was demonstrated in transgenic orange (*Citrus sinensis* L. Obs. cv. Pineapple) plants carrying a chimeric gene construct consisting of the cauliflower mosaic virus 35S promoter and the coding region of the tomato PR-5 protein. When challenged with *Phytophthora citrophthora* a significant reduction in lesion development was observed for the transgenic plants in comparison to the control plants not carrying the chimeric gene construct. The transgenic line also achieved plant survival rates significantly higher than control plants when transgenic trees were inoculated with oomycete cultures. These results provide evidence for the *in vivo* activity of the tomato PR-5 protein against *Phytophthora citrophthora*, and suggest that this may be employed as a strategy aimed at engineering *Phytophthora* disease resistance in citrus (Fagoaga *et al.*, 2001). Similarly, two rice Indica cultivars were transformed with the *tlp* gene construct containing the coding region of a thaumatin-like protein (TLP-D34), a member of the PR-5 group. Bioassays of the transgenic rice plants challenged with the sheath blight pathogen, *Rhizoctonia solani*, indicated that

over-expression of TLP resulted in enhanced resistance compared to control plants indicating that PR-5 is associated with resistance against the pathogen (Datta *et al.*, 1999).

Resistance Gene Loci Structure and Evolution

The structure of an R-gene locus has been described as either simple (single-copy) or complex (multiple copies of and R-gene) (Hulbert *et al.*,2001). A simple locus consists of a single R-gene which carries significant genetic variation in an allelic series. The allelic forms of a simple R-gene indicate that these genes were functional in the past or are interacting with unknown virulence factors (Hulbert *et al.*, 2001). Mutations, gene conversion or recombination events were found to be mechanisms used by the plant to create new specificities for various pathogens. These three mechanisms can result in the formation of novel R-genes (mutation, gene conversion or intragenic recombination) or a novel combination (intergenic recombination) of R-genes in the new haplotype with new recognition specificity. Of the three methods, intragenic recombination has been shown most often to create new specificities. Although most R-genes that undergo mutations, gene conversion or recombination are nonfunctional, there are a few well characterized examples of the creation of novel R-genes with new resistance specificities. For example, the *L* locus in flax is characterized as a simple locus and confers race specific resistance to *Melampsora lini* that (Bittner-Eddy *et al*. 2000). The *L* locus consists of thirteen alleles (*L*, *L1* to *L11*, and *LH*). Intragenic recombination between *L9* and suL10 resulted in the formation of RL10 that showed novel specificity (Luck *et al*., 2000, Hulbert *et al.*, 2001). The R-genes at the *L* locus often mispair during meiosis and recombine unequally due to the sequence duplication present in the LRR region of R-genes at this locus (Bittner-Eddy *et al*. 2000).

A complex R-gene locus consists of multiple homologous genes with detectable functions. The closely linked R-genes at complex resistance gene loci are arranged in tandem arrays which allow new combinations of R-genes to be generated through recombination. For example *Rp1* is one of the best characterized complex disease resistance loci. This locus consists of fourteen genes (*Rp1-A* to *Rp1-F* and *Rp1-H* to *Rp1-N*) that map to the short arm of chromosome 10 in maize (Hulbert 1997). Each gene represents an NBS-LRR gene family and can carry from 1 to more than 50 genes in each family that are arrange in tandem arrays (Hulbert 1997). Due to the structure of this locus, recombination events can lead to the formation of new haplotypes with the combined resistance of both parents and a novel R-gene with new recognition specificity. An intragenic recombination event between an *Rp1-D* and *Rp1-I* heterozygote resulted in four recombinant progeny haplotypes with a non-parental resistance specificity (Hulbert 1997). Conversely, an intergenic recombination event between *Rp1-J* and *Rp1-F* parental haplotypes resulted in nine recombinants with the combined resistance of both parents. All of the *Rp1-JF* recombinants carried the *Rp1-J* parental allele at the centromere and *Rp1-F* parental allele at the distal end indicating that the combined resistance was due an intergenic recombination event (Hulbert 1997). Therefore, the structure of complex disease resistance locus can facilitate mis-pairing during meiosis resulting in the creation of novel R-genes and the re-assortment of the R-genes into new haplotypes.

The evolution of plant R-genes is primarily affected by selection pressure imposed on the host-plant by the pathogen (Lehmann P. 2002). In case of direct recognition between the R-genes and *avr* genes, selection pressure is imposed on the pathogen carrying the *avr* gene to escape recognition. This is accomplished by mutations in the *avr* gene that avoid plant recognition, however do not affect the virulence function. Once the plant recognition is

overcome, then selection pressure acts on the plant in order to generate novel R genes with new recognition specificity that is capable of recognizing the new *avr* genes. This phenomenon is known as the "arms-race" between the plant and pathogen and has two potential outcomes. One is that the R-genes and *avr* genes involved in direct recognition are lost by the plant and pathogen due to directional selection for advantageous alleles. The other outcome is that both the plant and pathogen maintain high sequence diversity in order to generate new R and *avr* genes through point mutations, intra and intergenic recombination, gene conversion or unequal crossing over.

Diversifying selection is responsible for the high amino acid variation at the R and *avr* loci that show direct interaction. This variation is due to the pressure imposed by pathogen virulence and plant resistance respectively in an interactive way. Diversifying selection (constant escalation and diversification) is characterized with an elevated ratio of non-synonymous to synonymous substitutions leading to a change in amino acids. This is the most common model that predicts the rapid evolution of R genes to match the changes in the pathogen *avr* genes. An example to illustrate this is the *L* locus of flax that confers resistance against *M. lini* (Ellis *et al.,* 1999). The *L* locus of flax comprises of 11 R genes (including *L5*, *L6* and *L7*) that confer race specific resistance against *M. lini* carrying the corresponding *avr* genes (*avrL567*). The R proteins (*L5, L6* and *L7*) physically interact with the *avr* genes (*avrL567*) as seen in the yeast two hybrid assay (Ellis *et al.,* 1999). The presence of multiple *avr* genes and R genes indicate diversifying selection at the R and *avr* loci causes high levels of amino acid sequence polymorphism and is a result of the 'arms-race' between plant and pathogen (Xiao *et al.,* 2008).

Compared to direct recognition of the pathogen *avr* genes, there is an advantage for indirect detection of the pathogen, because any changes in the *avr* genes that do not alter its virulence fail to escape recognition by R genes. Therefore the likely outcome is deletion of the *avr* genes to avoid recognition, however if the virulence activity is essential for the pathogen, loss of *avr* gene may cause fitness penalty for the pathogen. Hence in order to maintain a balance between enhanced virulence and cost of keeping the *avr* gene in the pathogen population, natural selection causes balancing polymorphism at the *avr* locus. This phenomenon is known as balancing selection (trench warfare or ebb and flow of the same gene) (Xiao *et al.,* 2008). Similarly the presence of R gene in the plant is advantageous only in the presence of the corresponding avr gene and may cause a cost of resistance in the absence of the pathogen (Tian *et al.,* 2003). Therefore natural selection will favor balancing polymorphism at the R locus. Thus in both the plant and pathogen natural selection favors presence of balancing polymorphism of the corresponding R and avr genes for a very long time (Xiao *et al.,* 2008). Balancing selection is responsible for the presence of two R genes namely RPM1 and RPS5 in *Arabidopsis* that detect the Avr proteins by guarding the host targets (RIN4) of the Avr proteins (Mackey *et al.,* 2002, Shao *et al.,* 2003). *Arabidopsis* segregates for a functional and null allele at these two loci. Sequence analysis of these R genes indicates the presence of a simple but stable presence/absence polymorphism (Caicedo *et al.,* 1999, Mauricio *et al.,* 2003). Another example is the indirect recognition of AvrRpt2 by RPS2 in *Arabidopsis*. There is low genetic diversity with simple resistance/susceptibility allelism at the RPS2 locus (Axtell and Staskawicz *et al.,* 2003, Mackey *et al.,* 2003). Sequence analysis in the populations indicate that resistant and susceptible haplotypes have been maintained in the population over a long period of time, which is in agreement with

balancing selection (Tian *et al.*, 2003). This is due to intermediate disease pressure which causes the R gene to evolve and maintain partially functional R variants; hence it is possible that RPS2 divergent alleles may be partially functional. In case of indirect recognition of the R-Avr genes, it is possible that the R gene may evolve independently to detect the virulence function of the same Avr gene. For example, *RPM1* from *Arabidopsis* and *Rpg1* from soybean appear to have evolved independently to indirectly recognize the AvrB effector from *P. syringae* (Xiao *et al.*, 2008).

In R genes the fragments of protein that interact with a ligand are subject to strong selection pressure than those regions that have a structural role (Lehmann 2002). The ratio of nonsynonynous substitution (K_a, nucleotide substitutions that cause a change in the amino acids) to synonymous substitution (K_s, nucleotide substitutions that fail to cause a change in the amino acids) for these proteins is informative. The value of this ratio for most proteins is 1, which is in agreement with the functional constraint on amino acid replacement. However, in case of the LRR domain this ratio is greater than one, since the LRR domain of the R genes provides race specific recognition specificity and is under strong positive adaptive evolution (Lehmann 2002). This is essential for the LRR region to develop new recognition specificity in order to recognize the ever evolving pathogen. The strong positive selection for the LRR region was seen in the *Cf* gene family of tomato, where the ratio of K_a/ K_s was greater than 1.This suggested that the solvent exposed residues of the LRR domain play a role in pathogen recognition (Parniske *et al.*, 1997). In another example, the rice *Xa21* gene confers resistance to *Xanthomonas oryzae pv oryzae* in a race specific manner. Transgenic plants carrying six *Xa21* gene family members indicated that one member *Xa21D* displayed a resistance spectrum similar to that observed for *Xa21* but conferred only partial resistance

(Wang *et al.*, 1998). Nucleotide substitution in the LRR region of rice *Xa21* and *Xa21D* showed a greater number of nonsynonymous substitutions (Wang *et al.*, 1998). Thus diversity at the LRR domain is essential for recognizing, binding and defending the plant against a broad range of pathogens (Lehmann 2002).

Maize

Maize is one of the world's most important crop plants, with multibillion dollar annual revenue. It is the third most important source of food for humankind after rice and wheat and will potentially become the most important crop by 2020 (Rosegrant *et al.*, 2009). Maize is the world's most extensively grown crop with an annual production of 313 and 273 million tons in 2011 and 2012 (http://faostat.fao.org) respectively in United States. It is affected by an average of 100 pathogens but only a fraction of disease are present in a given location depending upon various factors and rarely do the number of these disease become severe. The most important and destructive diseases are leaf blights, stalk rots, ear and kernel rots, seedling diseases, smuts and sometimes bacterial and viral disease also cause economic losses to total production of maize crop.

There are numerous maize foliar diseases that affect maize. However gray leaf spot; northern corn leaf blight, southern rust and common rust have been reported as the most damaging foliar disease (Balint-Kurti and Johal 2009). Common rust, caused by *Puccinia sorghi*, is found wherever corn is grown and is favored by cool temperatures. Yield losses estimate 2 to 8% for every 10% of leaf area affected and are severe when infection begins earlier in the season or a susceptible hybrid is grown (Hooker, 1985). Besides poor yield, infected plants may be shorter, have shorter ears with reduced diameter, and have a higher

probability of stalk rot (Kim and Brewbaker 1976). Specific resistance against common rust is available in corn, and many *Rp* genes have been identified that confer resistance as a hypersensitive reaction. The resistant genotypes are characterized by the production of small chlorotic or necrotic flecks in response to the pathogen, which stops further infection. The same resistance reaction is seen at both the seedling and adult stage of the plant. Alleles at four or more gene loci located on chromosomes 3, 4, 6 and 10 have been identified that confer specific resistance and are simply inherited, usually in a dominant manner. The *Rp1* complex consists of 14 NBS-LRR genes (*Rp1-A* to *Rp1-F* and *Rp1-H* to *Rp1-N*) that confer race specific resistance against *Puccinia sorghi*. This complex contains duplications and is active in generating new sources of resistance through recombination (Richter *et al.,* 1995). The most widely used *Rp1D* gene, especially in sweet corn, had recently become effective in controlling the pathogen in the Midwestern and southern United States (Pate *et al.*, 2001).

In addition to the above mentioned pathogens the smut fungi are important agricultural pathogens responsible for significant crop yield losses. Yield losses due to corn smut in currently available partially resistant field varieties are kept below 2%. If one considers that maize is the most economically important crop in the USA, generating $79.8 billion in 2012 with approximately 97.2 million hectares planted (2013 World of Corn, National Corn Growers Association); even a 2% loss represents nearly $1.596 billion annually. Several methods are utilized to control corn smut disease including crop rotation, sanitation, seed treatments, application of foliar fungicides, modification of fertility and biological controls (Pataky and Snetselaar, 2006). However, host resistance is the only practical method of managing common smut in areas where *U. maydis* is prevalent. Currently, there are no known maize lines available that are immune to infection by *U.*

maydis (Allen *et al.*, 2011). This indicates that *U. maydis* is an important pathogen that causes significant losses on maize which necessitates the identification of new sources of resistance.

U. maydis is a hemibasidiomycete and has a very narrow host range. The disease induced in maize is known as "corn smut disease" or huitlacoche, the Nahuatl name by which it has been known in Mexico since ancient times. Disease development is characterized by chlorosis (yellowing of tissue), anthocyanin pigmentation (reddish-purple color), stunting, and especially tumor formation. The most conspicuous symptom of the disease caused by *U. maydis* is the formation of plant tumors by induction of cell proliferation in meristematic plant tissue (Banuett 1995). Tumors can develop on the leaves, stems, tassels and ears that can reach a large size, particularly on mature plants. The infected ears of corn are known as the culinary delicacy of huitlacoche (the "ambrosia of the gods"). Another characteristic symptom is that in the appropriate host genetic background, the induction of anthocyanin pigmentation (reddish-purple color) in the tumorous tissue and surrounding areas can be seen. The tumors that develop on the ears have the most severe effect on seed yield, as the fungus completely replaces the kernel tissue with masses of black spores (teliospores). Another symptom observed, particularly in the field, is the development of female flowers in the tassel (which normally only contains male flowers) and the development of tassel-like structures in the ear (which normally has only female flowers) (Banuett, 1995). *U. maydis*, in contrast to the other smut fungi, produces prominent symptoms on all aerial parts of the host plant. In practice, maize seedlings can be infected at the three leaf stage. In about one week the symptoms, in this case tumor formation, can be scored (Brefort *et al.*, 2009).

U. maydis exhibits three life forms in its life cycle a unicellular, uninucleate haploid form that is saprobic (sporodium), a parasitic dikaryotic filamentous form that is pathogenic and a diploid form (teliospores), which are formed only in the tumorous tissue. The fungus overwinters as teliospores in crop debris and in the soil, where it can remain viable for several years. In the spring and summer, teliospores germinate and produce basidiospores, which are carried by air currents or are splashed by water on to young developing tissues of corn plants. Basidiospores germinate and produce a hypha, which can enter epideremal cells directly. After the initial development, however its growth stops and the hypha usually wither and sometimes die, unless it contacts and fuses with haploid hypha derived from a basidiospore of the compatible mating type. If fusion takes place, the resulting hypha becomes dikaryotic, enlarge in diameter and grows into the plant tissues mostly intracellularly. Cells surrounding the hypha are stimulated to enlarge and divide, and galls begin to form. Galls in older plants seem to consistently result from local infections. Systemic infections occur occasionally in very young seedlings. However, only a small number of local infections develop into typical, large galls with the others remaining too small to be visible. The mycelium in galls remains intercellular during most of gall formation but before sporulation, the enlarged corn cells are invaded by the mycelium, collapse, and die. The mycelium utilizes the cell contents for its further growth. The gall then consists primarily of dikaryotic mycelium and plant cell remains. Most of the dikaryotic cells subsequently develop into teliospores and, in the process, seem to absorb and utilize the protoplasm of other mycelia cells, which remain empty. Only the membrane covering the gall is not affected by the fungus. During the final stages of infection the membrane breaks and the teliospores are released. Some of the released teliospores may cause new infections

and new galls during the same season if they land on young meristematic corn tissue. However, most of the teliospores fall to the ground or remain in the corn debris where they can survive for several years (George Agrios 1994).

Despite a substantial amount of effort currently there are no commercially available maize plants conferring increased levels of resistance against *U. maydis*. There are many reasons for this lack of success, including lack of adequate knowledge and clear understanding of the defense pathways that are activated in response to *U. maydis* infection. The sheer economic value of maize makes it an attractive target to study the differentially expressed gene in response to *U.maydis* infection, in order to gain a better understanding of plant pathogen interactions leading to activation of plant defenses thereby facilitating identification of new sources of resistance. The advantage of this would be the production of commercially available maize plants that have increased resistance against *U. maydis* and higher economic value due to the increased yield. Maize has been an attractive system for studying plant disease resistance genetics both as a model system and as a target in its own right. The fact that maize is such an important crop means that findings made in maize can be directly useful as well as provide a framework for disease resistance studies in other species. The impressive surge in the utilization of maize grain for biofuel production further underlines the urgency to improve the agronomic performance of maize to ensure that a suitable and sustainable domestic supply will not be outpaced by the rapidly expanding global demand for maize-derived food, feed and fuel.

Teosinte

Teosinte is the common name for the wild taxa of *Zea* and is the progenitor of Maize. The genus *Zea* is further divided into two section namely; *Luxuriantes* and *Zea. Luxuriantes* comprises three species: the first, species is *Z. diploperennis*; it is a ~2 to 2.5 meters tall diploid (*n=10*) perennial with a narrow distribution in the state of Jalsco, Mexico (Matsuoka *et al.,* 2002). The characteristic features of this species include 2-15 somewhat lax branches of tassels with trapezoidal fruitcase and long, slender, short tuberous rhizomes (Doebley and Iltis 1980). Second, Z. *perennis* is ~1.5 to 2 m tall tetraploid perennial (*n=2x=20*) with a narrow distribution in the state of Jalsco, Mexico (Reeves and Mangelsdorf 1959). It is the only polyploid in this genus which can be distinguished from *Z. diploperennis* due to the presence of 2-8 erect tassels branches (Reeves and Mangelsdorf 1959). The third, species in the section *Luxuriantes* of the genus *Zea* is the 3-4 meters tall annual *Z. luxurians,* a native to Guatemala, Honduras and Nicaragua. It is an annual consisting of 4-20 erect branches of tassel with a trapezoidal fruitcase. It lacks rhizomes and due to its outer glumes of male spikelets having numerous fine veins can be distinguished from the other *Zea* species (Doebley and Iltis 1980).

Fours subspecies constitute the section *Zea,* these are; (1) *Zea mays* L. ssp. *Huehuetenangensis,* a native to western Guatemala with a long life cycle. It is taller than all the other teosintes (~5m) and has a triangular fruitcase (Doebley and Iltis 1980). (2). *Zea mays* L. ssp. *parviglumis* is found in the valley along Nayarit to Oaxaca of Mexico (Doebley and Iltis 1980). It has a short life cycle and is 2-5m tall with green to weak red glabrous leaf sheaths and large number of tassel branches ranging from 20 to 100. Due to its small (5-8 mm) tassel spikelets (glume) is it known as "*parviglumis*" (Doebley and Iltis 1980). (3) *Zea*

mays L. ssp. *mexicana*, ~1.5 to 4 meters tall with 10-20 tassel branches and triangular fruitcases (Schrader). It has a short life cycle (4-6 months) with large tassel spikelets that distinguish it from ssp. *parviglumis* and *huehuetenangensis* (Doebley and Iltis 1980). (4) *Zea mays* L. ssp. *mays*, is the domesticated maize with a single stalk and numerous seeds on a single cob. Unlike teosinte these seeds are not enclosed in a hard fruitcase and are roughly circular or ovoid in shape. The tassels of maize and teosinte are highly similar however the ears of teosinte are smaller than that of maize (Doebley 1984).

In the seedling growth stage maize and teosinte look alike however the morphological differences are significant at maturity especially in the inflorescence. Maize consists of a single stalk terminating in a tassel and has two to five ears. The ears of maize consist of numerous naked yellow to white colored kernels arranged in multiple rows on a single cob. On the contrary, teosinte is highly branched with each branch terminating in long tassel and the cobs comprise 5-10 kernels each enclosed in a hard fruitcase (Matsuko *et al.*, 2002). Teosinte has a brittle cob, while maize forms solid ones that do not release their seeds.

Beadle in 1939, 1978, 1980 put forth "the teosinte hypothesis", according to which teosinte was regarded the progenitor of maize (Beadle 1939, 1978, 1980). This hypothesis was based on the experimental, anthropological, archeological, geographical and linguistic evidence. In order to prove his hypothesis Beadle grew 50,000 maize-teosinte F_2 plants and noticed that one in every 500 F_2 plants had ears similar to either maize or teosinte indicating that few genes (4-5 genes) are responsible for the difference in ear morphology. Further, Doebley and Stec (Doebley and Stec 1991, 1993) used QTL mapping to identify the regions underlying the morphological changes in the ear of maize and teosinte. In the maize-teosinte F_2 population they found that five to six regions had a strong effect on ear morphology.

Doebley's research group identified a candidate gene *teosinte branched 1* (*tb1*) for one of the QTL. Maize *tb1* mutants had long lateral branches terminating in tassels and morphologically resembled teosinte concluding that *tb1* gene controls apical dominance and is responsible for changes in plant architecture. Sequence analysis of the *tb1* gene from maize and teosinte showed that this gene was under positive selection during domestication (Doebley 2004). This hypothesis was further supported by numerous biological evidence published from 1970–1990. One of these is the molecular genetic studies that used protein (isozyme) and DNA marker technology to study the genetic similarity between maize and teosinte (Doebley 1990). These studies concluded that *Zea mays* L. ssp. *parviglumis* is indistinguishable from maize and is thus the direct wild progenitor of maize.

The "teosinte hypothesis" served as the basis for two different models for the evolution of maize from teosinte (Matsioka *et al.*, 2002).The first model assumes a single domestication event as the starting point of maize evolution followed by rapid diversification also known as the single domestication model. According to this model teosinte must have gone through a series of very rare mutations that eventually led to its transformation in maize (Doebley 1990, Iltis 2000). Contrary to this the multiple domestication model assumes that due to the significant morphological changes between teosinte and maize, teosinte must have undergone numerous domestication events leading to the formation of modern maize. In order to test these two models Matsuko *et al.*, in 2002 performed a phylogenetic analysis using microsatellites on maize and *Z. mays* tesointes (Matsuko *et al.*, 2002). These studies provided genetic evidence to support single domestication model as responsible for the evolution of maize from teosinte. Molecular dating of maize domestication with archaeological evidence conclude that maize was domesticated from teosinte no earlier than

10,000 years ago (Iltis 2000). Recent molecular phylogeny studies prove that maize originated from *Z. parviglumis* roughly 9000 years ago through a single domestication event in the central Balsas River of southern Mexico (Matsioka *et al.*, 2002). The diversity seen in maize is due to introgression resulting in the creation of novel phenotypes by incorporating alleles from outside populations through hybridization. This is possible since *Zea* species are outcrossing and introgression between maize and teosinte as well as between maize races is possible.

Forty maize-teosinte introgression lines were provided for this work by Dr. Sherry Flint-Garcia at the University of Missouri, Division of Plant Sciences, and USDA-ARS. These lines were created by backcrossing ten different teosinte (*ssp. parviglumis*) accessions into the maize *B73* background, creating 900 teosinte introgression lines. Each line is near-isogenic and has an average of 4% teosinte from random parts of the genome. The 900 NILs have been genotyped with ~768 SNPs each. Therefore regions that have been introgressed into each line are known. A "minimum tilling path" of one-hundred lines from one teosinte accession was created. The one-hundred lines used for this work contains almost the entire teosinte genome with each introgression line carrying a different region from the teosinte genome.

Use of wild progenitors to improve traits of cultivated crops

In the past, the increased demand for agricultural productivity was fulfilled by a combination of genetic improvements, greater farming inputs (such as fertilizers, pesticides and water) and cultivation of more land (Tanksley *et al.*, 1997). However due to the increase in population and the depletion of agricultural land, we will be unable to meet the agricultural

demands in the future. Additionally, yield loss due to plant diseases has had a significant impact on agricultural productivity. Thus, genetic improvement of crops is the most viable approach by which food production can attempt to keep pace with the anticipated growth of the human population.

The current strategy to improve resistance is by breeding resistance genes from a resistant cultivar into a susceptible cultivar. This involves making controlled crosses and screening the progeny lines for resistance reaction, in addition to validating the resistant phenotype by using molecular markers or gene expression analysis. The traditional approach is to cross the exotic germplasm (containing the resistance gene) with the cultivated species (lacking the resistance gene). This method is effective when the trait of interest is controlled by one or a few genes. The progeny lines are then screened for the resistance phenotype. This involves inoculating hundreds or thousands of plants with a given a pathogen. One or more resistance lines are identified and used to make further crossing experiments. This methodology is known as marker assisted selection and is the most effective way of pyramiding multiple genes in a single cultivar. Marker assisted selection is not only cost-effective and efficient; it is also amenable to automation and high throughput. It can be applied for the maintenance of recessive alleles in backcrossing pedigrees and for pyramiding of resistance genes (Hajjar and Hodgkin 2007). This is advantageous as compared to transgenic crop production since it involves transfer of genes from the same or closely related species.

The use of closely related species to introgress resistance genes is also another method of improving resistance of cultivar crops. However, this methodology has not been very successful. This methodology was first reported in wheat where the maize *Rp1-D* gene

was transferred to wheat and barley (Ayliffe *et al.*, 2004). The *Rp1-D* confers resistance against common rust in maize. Wheat and barley lines carrying this gene failed to show resistance against three rust isolates. This was because most of the transcript in wheat and barley appeared truncated and lack of function was due to improper expression of the gene. Another explanation for this failure was the absence of the corresponding *Avr* gene in the other *Puccinia* species that attack these cereals. However in a number of crops the known genes for disease resistance are being used up as they are released in cultivars and then overcome by new races of a pathogen. In addition to this cultivated species often lack genes required by the plant breeder particularly genes for disease resistance (Knott 1971). The limited genetic diversity of crops makes them vulnerable to disease and this jeopardizes the potential for sustained genetic improvement brought over long term. Plant breeding has been extremely successful at increasing the frequency of beneficial alleles for yield at many loci, thus breeders have continued to make crosses among closely related, high yielding varieties. However there have been very few studies to rationalize a search for yield-enhancing genes in low yielding ancestral crops (Tanksley *et al.*, 1997). Although wild germplasm is perceived to be a poor source for improvement of most traits based on phenotypic examination, it is possible that most favorable genes (e.g. disease resistance) may be buried in them, only if these could be found they can be employed for crop improvement. Thus wild species are becoming increasingly important sources of germplasm in the breeding of many crops.

The transfer of resistance genes into cultivated crops from wild progenitors has been an important tool for improving disease resistance (Knott and Dvorak 1976). Previous studies report that over 100 beneficial traits have been derived from approximately 60 wild species

and incorporated in 13 cultivated crops (Hajjar and Hodgkin 2007). Out of these 100 beneficial traits over 80% were associated with pest and disease resistance (Hajjar and Hodgkin 2007). Of these 13 crops with incorporated wild genes in cultivated species, all except barley and chickpea have disease resistance genes derived from the wild progenitor (Hajjar and Hodgkin 2007). However, only maize, banana and groundnut have disease resistance as the only beneficial trait derived from the wild progenitor (Hajjar and Hodgkin 2007). In the early 1980 only a few examples of wild genes preventing devastation by pests and disease were seen, these include *Oryza nivara* providing resistance to grassy stunt virus in rice, *Solanum demissum* providing resistance to potato late blight, *Agropyron elongatum* and *Aegilops umbellulata* providing resistance to stem and leaf rust in wheat. In addition to these there, are many tomato disease resistance genes introgressed from wild species, mostly from *Lycopersicon pimpinellifolium* (Prescott-Allen 1986).

Resistances found in tomato wild relatives have been reported at a rate of about one per year since 1982 (Rick and Chetelat 1995) and over 40 resistance genes have been derived from *Lycopersicon peruvianum, L. cheesmanii pennelli* and several other wild relatives (Rick and Chetelat 1995). In rice *Oryza nivara* genes still provide strong and extensive resistance to grassy stunt virus to rice in south and south-east Asia (Barclay 2004). In potato, several new resistance genes that confer resistance to potato late blight are obtained from *Solanum demissum* and *Solanum stoloniferum* (Hajjar and Hodgkin 2007). In wheat, *Aegilops tauschii* has been used to improve resistance to Hessian fly, a major insect pest in the USA (Suszki 2005). In sunflower, disease resistance genes against downy mildew, rust, Verticillum wilt and broomrape have been incorporated from wild *Helianthus annuus* L. and *H. praecos* (Hajjar and Hodgkin 2007).

Apart from disease resistance wild relatives are also used to increase yield; however there are only a few examples of these since wild relatives have poor agronomic performance. This is also one of the reasons for using wild progenitor genes to improve resistance. An example to show that wild progenitor was used to improve resistance is seen in the chickpea cultivar 'BG1103' developed by the India Agricultural Research Institute having drought tolerance and temperature tolerance derived from *Cicer reticulatum* (S.Yadav *et al.,* 2002) This cultivar yield was 40% more than that of the competing cultivars but the increased yield is due to wild genes conveying increased drought and temperature tolerance, rather than specifically targeting yield (Hajjar and Hodgkin 2007). Another example of using a wild progenitor to increase yield is the production of hexaploid wheat by crossing durum wheat and the wild relative *Aegilops tauschii*. These lines were then backcrossed to an elite bread wheat cultivar to produce wheat with superior quality, disease resistance and yield. In addition to improving yield wild relatives are being used to improve the quality of cultivated crops. This is seen in tomatoes that have improved quality traits such as increased soluble solid content, fruit color and adaptation to harvesting obtained from the wild cultivar *Lycopersicon pimpinellifolium* (Hajjar and Hodgkin 2007). In tomato, lines have been created that contain specific QTLs from the wild species *lycopersicon esculentum* that outperform the original cultivated species by 48, 22, 33 % of yield and soluble solid content and fruit color respectively (Tanksley and McCouch 1997). The magnitude of these improvements is substantial as compared to 1% improvement achieved through traditional breeding. The drastic improvement in the red fruit color of tomato is due to the pigment lycopene. The wild tomato lacks an active enzyme required for the last step in the pathway for synthesis of lycopene, hence they cannot synthesize lycopene and the fruit remains green

even after it is ripe. However the wild tomato contains genes that can enhance the earlier steps associated in the biosynthetic pathway of lycopene production. This wild tomato when crossed with the cultivated species containing the active form of the gene for lycopene synthesis leads to higher levels of the pigment production in the interspecific offspring (Tanksley and McCouch 1997).

Though the use of wild progenitor genes to improve traits of cultivated crops is being employed on a vast scale, it has not been successful always. There are many limiting factors to this which include difficulties with interspecific crossability, blocks to hybridization and hybrid sterility and retention of undesirable agronomic traits. An example of this is the crosses of cowpea cultivars with *Vigna unguiculata* (L.) Walp suspp. *dekindiana* and *pubescens* which failed to produce any breeding lines with high agronomic performance or better quality traits (Hajjar and Hodgkin 2007). In addition to these efforts to reduce deleterious effects of cross breeding with wild relatives through backcrossing this method is costly and time-consuming, and will no doubt affect the speed with which new cultivars are released. Further, molecular techniques offer a partial solution but there will likely continue to be cases where pleiotropic effects limit the use of genes from wild relatives. Despite the continuing steady increase in use of wild progenitor genes to improve the traits of cultivated crops, there is a high probability to expect a greater increase of this methodology in the future. This is due to the increased knowledge of the genetics of desired traits, increased availability of wild relatives in genebanks, improved inter-specific hybridization capabilities and advances in molecular technologies.

Objectives

The specific objectives of this research were:

1. Identify and characterize new sources of resistance from teosinte and maize-teosinte introgression near isogenic lines (NILs) in response to pathogen (*U. maydis*) infection
2. Characterize the expression pattern of differentially expressed genes in maize in response to pathogen (*U. maydis*) infection

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CHAPTER 2

TRANSCRIPTOME ANALYSIS OF MAIZE IN RESPONSE TO *USTILAGO MAYDIS*, THE CAUSAL AGENT OF CORN SMUT[1]

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Abstract

Ustilago maydis (*U. maydis*), the causal agent of corn smut is an important agricultural pathogen that is responsible for significant yield losses of approximately $1.24 billion annually in the United States. Several methods including crop rotation, sanitation, fungicide application, modification of fertility, biological control and seed treatments are currently used to control corn smut. However, host resistance is the only practical method for managing corn smut. There are currently no maize lines available that confer complete resistance to *U. maydis.* To identify genes associated with resistance to corn smut, transcriptome profiling was conducted on maize genotypes demonstrating resistance and susceptibility to *U. maydis.* Among the 17, 555 genes monitored using an Affymetrix GeneChip maize genome array, 5,639 genes showed significant differential expression between the control and *U. maydis* inoculated maize lines at 24 hours post inoculation (hpi). From this data set, 529 genes were up-regulated (≥1.5 fold change), whereas 5,110 were down regulated (≤ 1.5 fold change) in inoculated resistant and susceptible maize plants, respectively. The up-regulated genes were grouped into 7 categories, and were classified as genes coding for defense related proteins, enzyme families, receptor like kinases, photosynthesis, regulation , metabolism and transcription. The down regulated genes were grouped into 10 categories representing genes involved in enzyme families, hormones, plant glycolysis, photosynthesis, metabolism, cell function, transcription, defense related genes, receptor like kinases and regulation overview. These findings were confirmed by quantitative real time polymerase chain reaction (qRT-PCR) with 5 randomly selected genes from defense related genes categories. These findings provide insight into the complexity of

biotrophic interactions in an incompatible interaction and indicate that the activation of plant defenses in response to *U. maydis* infection is similar to other biotrophic interactions.

Introduction

A number of biotic and abiotic factors continue to challenge maize production. At present, one of the threats to this crop is corn smut caused by *Ustilago maydis* (*U. maydis*), a member of the fungal phylum Basidiomycota. Hot and dry weather conditions are favorable for *U. maydis,* which can attack maize during its early stages of development. However, corn smut occurs more frequently on maize ears, tassels, and nodes than on leaves, internodes, and aerial roots. *U. maydis* is a biotrophic pathogen, therefore it depends on the survival of colonized host cells. Early disease symptoms are macroscopically visible 12 to 24 hours after inoculation and include chlorosis and small necrotic spots at the site of infection. The plant recognizes *U. maydis* hyphae during intracellular development in the epidermal layer during cell to cell movement. Usually, the colonized plant cells remain alive, whereas plant cells containing older hyphae that lack cytoplasm undergo cell death. During the later stages of infection, *U. maydis* induced tumors are formed by the enlargement and proliferation of plant cells. Large fungal aggregates are formed in the tumors. This occurs without the elicitation of programmed cell death (Hypersensitive Response, HR) in the surrounding plant tissue (Doehlemann *et al.* 2008). Induction of tumor growth is also accompanied by accumulation of anthocyanins resulting in a red pigmentation of the infected tissue (Brefort *et al.* 2009). Currently there are no maize cultivars that are completely resistant to *U. maydis* and no single gene that confers resistance to the pathogen has been identified (Smith, J.T., 2011). Partially resistant maize cultivars are capable of maintaining yield losses up to 2% (Allen, A., *et. al*. 2011). However, even a 2% loss of 62 billion dollars is equivalent to 1.24 billion

dollars annually. To control corn smut disease, several methods have been recommended, including crop rotation, sanitation, seed treatments, application of foliar fungicides, modification of fertility, and biological controls. However, host resistance is the only practical method of managing corn smut in areas where *U. maydis* is prevalent (Smith, J. T., 2011). Despite efforts to control plant diseases, adverse environmental conditions and the emergence of resistant pathogen strains make it difficult to control plant diseases, including corn smut (McDowell *et al.* 2003). Due to years of co-evolution with pathogens, plants have evolved complex mechanisms to defend themselves from disease (McDowell *et al.* 2003). Some defense responses are constitutive, while others are induced upon pathogen attack. Induction of plant defenses involves a wide variety of biochemical events that are triggered rapidly and coordinately during a given plant-pathogen interaction. The first step in the activation of plant defense responses involves recognition (directly or indirectly) of the pathogen avirulence (*Avr*) gene protein(s) by the plant resistance gene (R-gene) protein(s). This recognition leads to an elaborate induction process resulting in the activation of plant defenses. It has been shown that the activation of plant defenses in the *U. maydis*-maize interaction includes the induction of pathogenesis related (PR) genes, production of secondary metabolites as well as the reinforcement of the plant cell wall (Doehlemann *et al.* 2008).

Induction of PR proteins in various plant tissues is one of the major biochemical events that occurs when plant defenses are activated (Van Loon, 1997). PR proteins have been characterized in many plant species and are currently grouped into 17 families designated PR-1 through PR-17 (Gorlach *et al.*, 1996, Van Loon 1999, Okushima *et al.*, 2000, Christensen *et al.*, 2002). The characteristic function of the majority of the PR proteins

is proteinase-inhibition, membrane permeability and hydrolytic activity which are classic antifungal, antibacterial, nematicidal, antiviral and insecticidal properties (Van Loon and Van Strien, 1999; Van Loon, 2001; Selitrennikoff, 2001). Induction of PR proteins is achieved through the activation of many signaling pathways, including different receptors or chemical elicitors such as salicylic acid (SA), ethylene (ET), jasmonic acid (JA) and systemin (Ward *et al*., 1991; Xu *et al*., 1996; Maleck *et al*., 2000; Campos *et al*., 2002). It has been shown that in a resistant reaction, activation of plant defenses is followed by a cascade of signaling events inside the plant cell culminating in the activation of PR genes (Maleck *et al*., 2000; Campos *et al*., 2002). Conversely, susceptibility is correlated with very low or undetectable levels of PR gene expression. This indicates that the activation of PR genes is directly associated with resistance and serves as a molecular marker for a resistance response.

Analysis of the genes differentially expressed in resistant and susceptible cultivars has lead to the identification of candidate genes directly related to resistance (Baldwin *et al.*, 1999, Nadimpalli *et al.*, 2000, Maleck *et al.*, 2000, Schenk *et al.*, 2000). This is a crucial step in the development of resistant cultivars. However, identification of candidate genes associated with resistance has been a difficult task because most plant species carry a large number of R-genes and often express a variety of genes capable of adversely affecting the pathogens ability to cause disease. Therefore, studying genes differentially expressed in maize in response to *U. maydis* inoculation will identify candidate genes for resistance. The specific objectives of this study were to identify and analyze the genes differentially expressed in resistant and susceptible maize cultivars in response *U. maydis* inoculation and to better understand the mechanisms of resistance to *U. maydis*.

This study provides insights into the genes that are contributing to resistance, thus generating information regarding the mechanisms of resistance of which little is known. These studies are important for the design of novel strategies to develop maize cultivars with improved resistance to *U. maydis*. The objectives of this study were to: 1) Characterize the expression pattern of differentially expressed genes in maize in response to pathogen (*U. maydis*) infection.

Materials and Methods

Plant material

Four *Zea mays* near-isogenic lines (NILS) were used for this study. Seed for all of the lines were obtained from Germplasm Resources Information Network, USDA (GRIN); (1) *Zea mays subsp. mays* NSL 30060 (United States, Wisconsin), (2) *Zea mays subsp. mays* Ames 22443 (United States, Minnesota), (3) *Zea mays subsp. mays* Ames 27104 (United States, Iowa) and (4) *Zea mays subsp. mays* PI 511562 (United States). The four maize lines were selected based on their geographic origin in the Midwestern states where maize production is the highest in the U.S and because of their potential for resistance to *U. maydis*. The four maize lines are used in several breeding programs in the Midwestern states and are resistant to several important pathogens. However, resistance to *U. maydis* was unknown for the four maize lines.

Plant inoculations and RNA isolation

The four maize lines (NSL 30060, Ames 22443, Ames 27104 and PI 511562) were used for *U. maydis* inoculations in this study. The experiment was conducted using the four maize lines with two factors; 1. Resistance or susceptibility and 2. Inoculated or mock

inoculated. Experimental units were single seedlings with six seeds for each line. Seeds were planted in flats and placed in a growth chamber with day and night environments of 28/20°C temperature and 12/12 h of photoperiod, respectively and approximately 500 µmol m^{-2} sec^{-1} photosynthetically active radiations at the top of the canopy. The relative humidity was maintained during the day and night at approximately 70% and 90%, respectively. All plants were kept in the same growth chamber to maintain a growth environment that is congruent across the experiment.

Wild type *U. maydis* strain ½ (mating type *a1b1*) and a near isogenic strain 2/9 (mating type a2b2) were used for inoculations. Both strains were grown separately in potato dextrose broth at 30^0C to an OD_{600} of 1.0 (~ 1 X 10^7 cells/ml). Cells were suspended in water to a final concentration of 1 X 10^6 cells/ml. Seven days after planting, the culm of each plant was injected just above the soil line with ~100µl of cell suspension containing 1 x 10^6 cells/ml of the wild type *U. maydis* strains. The control plants were mock inoculated with water only. After inoculation, the plants were returned to the growth chamber until phenotypic scoring and tissue sample collection for RNA extractions. The seedlings were scored each day for 21 days beginning 7 days post inoculation (dpi) to monitor the disease progress and identify resistant lines (Chavan and Smith 2014). A standard disease rating system for *U. maydis* was utilized to score the plants with a 0 (highly resistant) to 5 (highly susceptible) infection type rating scale. Intermediate scores include 1C (chlorosis), 1A (anthocyanin pigmentation), 2 (leaf galls), 3 (stem galls) and 4 (basal galls). Three independent experiments were performed consisting of six seedlings for each line. The maize line that demonstrated the highest level of resistance in all three resistance reaction screening experiments was used for RNA extraction and microarray analysis.

Total RNA was isolated from 1 gram of leaf tissue collected from the resistant maize plants using the TRIzol® reagent as described by the manufacturer (Invitrogen, Carlsbad, CA). RNA was isolated from each seedling at 12 and 24 hours post inoculation (hpi) and 2 days post inoculation (dpi). Sampling time was selected based on previous studies and their similarities to the experiments described for this work (Doehlemann *et al.* 2008). The RNA was further purified with Qiagen (Valencia, CA) RNeasy Mini Kit columns to obtain high quality RNA samples. The quantity and quality of the RNA samples were evaluated using a Nanodrop spectrophotometer, agarose gel electrophoresis and a Bioanalyzer 2100.

Microarray hybridization and data analysis

The expression pattern of PR genes from the maize line that demonstrated the highest level of resistance in response to *U. maydis* and the susceptible maize line was determined with Affymetrix GeneChip® Genome Array technology (Santa Clara, CA). Maize Genome Array processing, including hybridization of probes, staining, washing and uploading data was performed at Kansas State University, Gene Expression Facility. An Agilent (Agilent Technologies, Palo Alto, CA) Bioanalyzer 2100 was used to first test the RNA quality. The Affymetrix (Santa Clara, CA) One-Cycle Target Labeling and Control Reagent were used to synthesize 1st strand cDNA from total RNA (5µg) isolated from each biological replication, following the manufacturer's instructions. This was done for RNA isolated from resistant and susceptible maize seedlings. Synthesis and labeling of cDNA/cRNA were performed using Affyinity Script cDNA Synthesis Kit (Agilent Technologies, Palo Alto, CA) The resulting labeled cRNA (15µg) was fragmented and hybridized to GeneChip® Maize Genome Array using Affymetrix GeneChip® Array technology (Affymetrix, Santa Clara, CA). Three microarray chips were hybridized to three separate biological replicates. A total of twelve

microarrays were used for this work. Three microarray chips were used for each resistant inoculated, resistant control, susceptible inoculated and susceptible control RNA sample.

Expression data CEL files were imported into the JMP Genomics 4.1 software (Bioinformatics Center, Kansas State University) to scale the average signal intensity of each chip to 500. To further normalize the data, log2 data transformation, Kernel Surface background correction, and Quantile data normalization programs were applied to the expression data. ANOVA (Analysis of Variance) significance test at a p-value < 0.05 and an expression fold change ≥ 1.5 and ≤ 1.5 cutoff level was applied to the four treatment comparisons. The four treatment comparisons were: (1) resistant inoculated (RI) with resistant control (RC), (2) resistant inoculated (RI) with susceptible inoculated (SI), (3) susceptible inoculated (SI) with susceptible control (SC) and (4) resistant control (RC) with susceptible control (SC). Genes demonstrating a ≥ 1.5 fold change in expression in comparison to the control were considered to be up-regulated, whereas genes with a fold change ≤ 1.5 were considered to be down-regulated. The differentially expressed genes (up-regulated and down-regulated) obtained from the microarray data were classified into different categories based on their function using the MapMan tool. After classifying the genes based on their function, expression values for the genes in the four different treatments was compared. The genes that showed the highest level of expression in the RI-SI treatment as compared to the remaining three comparisons (RC-SC, RI-RC, SI-SC) were analyzed. Additionally, genes that were identified in each comparison were analyzed to determine the function of each gene and their potential role in plant defenses. This was done by comparing the predicted function of each gene with the function of known genes described in an incompatable interaction during a biotrophic interaction.

Real-time PCR was used to confirm the differential expression of the genes identified from the microarray analysis. Five genes that were either up or down regulated (differentially expressed) were selected and used for the real-time PCR differential expression verification. The differentially expressed genes were selected based on their functional identities and expression profiles generated from the MapMan program. Genes of interest were selected based on functional identities that correlated with plant defenses. Sequences obtained from the microarray data were utilized to design gene-specific primers for the genes of interest that were differentially expressed in the resistant and susceptible maize lines. Gene-specific primers were used to PCR amplify genomic DNA isolated from the resistant (NSL 30060) and susceptible (PI 511562) maize lines. The resulting PCR products were gel purified, cloned into the Invitrogen (Carlsbad, CA) TOPO TA cloning vector and sequenced to verify that the gene-specific primers amplified the correct genes. The same gene-specific primers were then used to perform real-time PCR on RNA samples isolated from the two maize lines that were mock inoculated (RC-SC) and inoculated with *U. maydis* (RI-SI). First strand cDNA was synthesized from the RNA samples using Oligo-dT primers from the StrataScript First-Strand Synthesis System (Stratagene) as described by the manufacturer. Real-time PCR (qPCR) was performed with the Bio Rad iQ5 Optical System (Bio-Rad) using the iQ SYBR Green Supermix (Bio-Rad). Each 20 µl PCR reaction mixture contained: (1) 6 µl of RNAse free water, (2) 1 µl of 5000nM each forward and reverse primer, (3) 10 µl of SYBR green supermix and (4) 20ng of cDNA. PCR reaction conditions were as follows: (1) Template denaturation (95°C for 3 min), (2) Template amplification and quantification (40 cycles at 95°C for 10 second, 60°C for 30 s, 72°C for 30 s, 1 cycle at 72°C for 10 mins.), (3) Melt curve analysis (83 cycles at 60-95°C with a heating rate of 0.5°C/s), (4) 95°C for 5 s, (5)

Extension (60°C for 30 s) and (6) Template cooling at 4°C forever. Two biological replicates and a negative control without the cDNA template were run with each sample. The positive (internal) controls used were GADPH and 18sRNA. A total of 5 primers were designed for amplicons ranging from 200-400 bp in size for each gene (Table 2.1).

Results

Phenotype scoring

One maize line was identified (NSL 30060) that demonstrated a disease rating score ranging from 1-2 (Figure 2.1a thru 2.1c). This line showed chlorosis, anthocyanin pigmentation and minute leaf galls. The leaf galls were not clearly visible and could be felt only by touching the leaf blade. The minute leaf galls either disappeared or did not enlarge in size as the plant matured. As a result, the NSL 30060 line was characterized as resistant. Conversely, the remaining three maize lines (Ames 22443, Ames 27104 and PI 511562) demonstrated a disease rating score ranging from 3-5 (Figure 2.2a and 2.2b). The plants initially showed large leaf, stem and basal galls. The plants with leaf galls later developed stem and/or basal galls. The plants with stem or basal galls subsequently died as the disease progressed. None of the plants reached maturity. Therefore, Ames 22443, Ames 27104 and PI 511562 were characterized as susceptible lines.

Differentially expressed genes

The effects of *U. maydis* inoculation on gene expression in resistant and susceptible *Zea mays*, lines was determined by comparing the expression profile data from inoculated and mock-inoculated treatments at 24 hours post inoculation (hpi). Four different comparisons were made: 1. Resistant Inoculated (RI) vs. Susceptible Inoculated (SI), 2.

Resistant Control (RC) vs. Susceptible Control (SC), 3, Resistant Inoculated (RI) vs. Resistant Control (RC), and 4. Susceptible Inoculated (SI) vs. Susceptible Control (SC).

A total of 5,639 genes from the RI-SI genotypes were differentially expressed at $p \leq 0.05$ (Table 2.1). The up-regulated genes were assigned to 7 functional categories (Table 2.3) and the down-regulated genes were assigned to ten functional categories (Table 2.4). There were 7 categories (Defense related, enzyme families, metabolism, photosynthesis, receptor-like-kinase, regulation overview, transcription) that were common in both up and down-regulated genes, however there were an additional 3 (cell function, hormone, plant glycolysis) categories that were seen only in the down regulated genes (Table 2.4). Therefore, all of the up-regulated and down-regulated were grouped in to 10 functional categories and are described below.

Classification of differentially expressed genes;

1. Defense related genes

Genes involved in defense response, biotic stress, pathogenesis related genes, resistance protein, stress response, cell death, degradation of exogenous proteins, oxidation, reactive oxygen species, MAP kinases and other cell rescue activities were grouped under this category, which formed the largest group for the up-regulated genes (Table 2.3 and Table 2.5). In the RI-SI comparison, 106 of the 230 up-regulated genes were classified into the defense related genes category. The 106 genes ranged from 9.05 to 1.52 ($-\log_{10}$(p-value) in expression values. Similarly, 96 of the 228 up-regulated genes identified in the RC-SC comparison were grouped in the defense related genes category with expression values ranging from 9.18 to 1.37 ($-\log_{10}$(p-value). Functions for 31of the 51 up-regulated genes in the SI-SC comparison were associated with defense related functions with expression values

ranging from 6.40 to 1.33 ($-\log_{10}$(p-value). The RI-RC comparison identified 2 up-regulated genes in this category with the expression values ranging from 2.64 to 1.85 ($-\log_{10}$(p-value).

In contrast to the large number of up-regulated genes observed in the defense related genes category, there were fewer down-regulated genes identified in the defense related genes category for the four comparisons (Table 2.4 and Table 2.6). The RI-SI comparison indicated that only 12 of the 5,110 down-regulated genes were grouped in the defense related genes category and ranged from 5.00 to 3.48 ($-\log_{10}$(p-value) in expression. Similarly, the RC-SC comparison demonstrated that 38 of the 4,872 down-regulated genes were defense related with expression values ranging from 4.53 to 1.31 ($-\log_{10}$(p-value). Only 5 of the 2,319 down-regulated genes were present in the defense related genes category from the SI-SC comparison with expression values ranging from 3.59 to 2.56 ($-\log_{10}$(p-value). Similarly, 11 of the1,162 down-regulated genes from the RI-RC comparison were associate with defense related gene function and ranged from 3.46 to 1.36 ($-\log_{10}$(p-value) in expression.

2. Enzyme families

The enzyme families category include genes with the following function; cytochrome P450, oxidases, nitrilases, glutathione-S-tranferase, UDP-glycosyltransferases, acetyltransferases, alcohol dehydrogenase, O-Methyltransferases, phospatases, peroxidases and glucosidases (Table 2.3 and Table 2.5). Twenty-two of the 230 genes were up-regulated in the RI-SI comparison. The expression values ranged from 9.84 to 1.54 ($-\log_{10}$(p-value) for the 22 up-regulated genes. For the RC-SC comparison, 30 of the 228 up-regulated genes identified as enzyme families showed expression values ranging from 9.64 to 1.40 ($-\log_{10}$(p-value). Similarly, only 4 of the 51 genes from the SI-SC comparison were classified in this category with expression values ranging from 5.34 to 1.38 ($-\log_{10}$(p-value).

The down-regulated genes from the four comparisons were also analyzed for the enzyme family category (Table 2.4 and Table 2.6). The comparisons identified 37 genes with expression values ranging from 5.41 to 2.10 ($-\log_{10}$(p-value) in the RI-SI comparison, 256 genes with expression values ranging from 5.42 to 1.30 ($-\log_{10}$(p-value) in the RC-SC comparison, 270 genes with expression values ranging from 4.28 to 1.37 ($-\log_{10}$(p-value) in the SI-SC comparison and 75 genes with expression values ranging from 3.83 to 1.32 ($-\log_{10}$(p-value) in the RI-RC comparison.

3. Metabolism

Genes associated with metabolism of compounds such as amino acids, lipid metabolism, nitrogen, sulphur, nucleotides, fatty acids, carbohydrates, mitochondrial-e-transport and secondary metabolism were grouped under this category (Table 2.3). In the RI-SI comparison, 35 of the 230 up-regulated genes were associated with metabolic processes and ranged from 7.90 to 1.48 ($-\log_{10}$(p-value) in expression. The RC-SC comparison identified 47 of 229 genes that were up-regulated and ranged from 10.37 to 1.39 ($-\log_{10}$(p-value) in expression. Only 10 of the 51 genes in the SI-SC comparison were classified in the metabolic processes category and ranged from 6.36 to 3.37 ($-\log_{10}$(p-value) in expression.

There were more down-regulated genes identified in the metabolism category than up-regulated genes (Table 2.6). In the RI-SI comparison, 728 of 1,965 down-regulated genes were associated with metabolism and ranged from 6.57 to 1.30 ($-\log_{10}$(p-value) in expression. The RC-SC indicated that 449 of the 1,786 down-regulated genes were associated with metabolism and ranged from 6.34 to 1.30 ($-\log_{10}$(p-value) in expression. Similarly, 202 of the 2,319 down-regulated genes identified in the SI-SC comparison demonstrated expression values ranging from 4.94 to 1.31($-\log_{10}$(p-value). The RI-SC comparison indicated that 105

of the 1,162 down-regulated genes were associated with metabolism and ranged from 4.08 to 1.30 ($-\log_{10}$(p-value) in expression value.

4. Photosynthesis

There were very few differentially expressed genes identified in the four categories with functions associate with photosynthesis and greater than 1.5 fold differential expression (Table 2.5). Five of the 230 up-regulated genes from the RI-SI comparison ranged from 7.72 to 2.18 ($-\log_{10}$(p-value) in expression. Six of 229 up-regulated genes from the RC-SC comparison ranged from 8.37 to 1.51($-\log_{10}$(p-value) in expression. Similarly, only 2 of the 51 genes identified in the SI-SC comparison were associated with photosynthesis and expression values ranged from 1.97 to 1.90 ($-\log_{10}$(p-value).

Concersely, a greater number of genes were identied that demonstared less than 1.5 fold difference in expression and had functions associated with photosynthesis (Table 2.6). For example, 50 of the 1,965 down-regulated genes from the RI-SI comparison were classified in to this category and ranged from 4.27 to 1.30 ($-\log_{10}$(p-value) in expression. Similary, 40 of the 1,786 genes down-regulated in the RC-SC comparison ranged from 4.80 to 1.37 ($-\log_{10}$(p-value) in expression, 10 of the 900 down-regulated genes from the SI-SC comparison ranged from 3.10 to 1.31 ($-\log_{10}$(p-value) and 7 genes of the 386 genes from the RI-RC comparison ranged from 2.79 to 1.33 ($-\log_{10}$(p-value) in expression.

5. Receptor-like-kinases

The receptor-like-kinase catergory included different kinases such as, receptor-like-cytoplasmic kinases, signaling receptor kinases, proline extension like kinases and leucine rich repeat kinases (Table 2.5). Of the 230 up-regulated genes identified in the RI-SI comparison, 5 genes were from the receptor-like-kinase category and ranged from 6.23 to

1.34 ($-\log_{10}$(p-value) in expression. Nine of the 229 up-regulated genes in the RC-SC comparison were grouped in the enzyme families' category and had expression values ranging from 6.90 to 1.42 ($-\log_{10}$(p-value). Only 1 of the 51 up-regualted genes from the SI-SC comparion was identifed as a receptor-like-kinases and demonstared a 3.86 ($-\log_{10}$(p-value) expression value.

From the RI-SI comparison, 138 of the 1,965 genes were down-regulated in the receptor-loke-kinase category and ranged from 7.37 to 1.30 ($-\log_{10}$(p-value) in expression (Table 2.6). The RC-SC comparison indicated that 73 of the 1,786 down-regulated genes were also associated with recptor-like-kinase function and ranged from 4.85 to 1.31($-\log_{10}$(p-value) in expression. Only 27 of the 386 down-regulated genes for the RI-RC comparison were receptor like-kinaes ranging from 4.30 to 1.30($-\log_{10}$(p-value) in expression. Sixteen of the 900 genes from the SI-SC comparison with predicted receptor-like-kinase functions range from 2.94 to 1.30 ($-\log_{10}$(p-value) in expression.

6. Regulation overview

The genes that were annotated with functions associated with protein modification, protein degradation and calcium regulation were classified in to the regulation overview category (Table 2.5). In this category, 26 of the 230 up-regulated genes where identified in the RI-SI comparison and ranged from 6.59 to 1.31 ($-\log_{10}$(p-value) in expression. Fifteen of the 229 genes from the RC-SC comparison with expression values ranging from 5.95 to 1.50($-\log_{10}$(p-value) were grouped in this category. Only one of the 51 up-regulated genes from the SI-SC comparison was identified in regulation overview catergory and demonstarted an expression value of 2.74 ($-\log_{10}$(p-value).

For the down-regulated genes, 49 of the 1,965 genes from the RI-SI comparison were identified in the regulation overiew catergory and ranged from 4.32 to 1.36 ($-\log_{10}$(p-value) in expression (Table 2.6). Additionally, 70 of the 1,786 genes from the RC-SC comparison were down-regulated in this category. The expression values for these genes ranged from 7.40 to 1.31 ($-\log_{10}$(p-value). Only 5 of the of 900 down-regulated genes from the SI-SC comparison had functions associated with regulation overview and ranged from 4.25 to 2.67($-\log_{10}$(p-value) in expression. The RI-RC comparison indicated that 10 of the 386 down-regulated genes were in the regulation overview catergory and ranged from 3.34 to 1.30 ($-\log_{10}$(p-value) in expression.

7. Transcription

Genes associated with RNA synthesis, RNA regulation, RNA processing, RNA modification and nucleotide synthesis were classified in to the transcription category (Table 2.5). Thirty-one of the 230 genes from the RI-SI comparison with >1.5 fold difference in expression were identified in this category and ranged from 8.98 to 1.61($-\log_{10}$(p-value) in expression. Twenty-six of the 229 up-regulated genes from the RC-SC comparion were grouped in the transcription category and demonstrated expression values from 9.50 to 1.56 ($-\log_{10}$(p-value). Only 2 of the 51 up-regulated genes from the SI-SC comparison had functions associated with transcription. The expression value for the two genes ranged from 3.96 to 2.30 ($-\log_{10}$(p-value).

The number of genes that showed <1.5 fold difference in expression and were functionally annotated in the transcription category varied in number (Table 2.6). For example, 353 of the 1,965 genes from the RI–SI comparison were down-regulated and ranged from 7.25 to 1.30 ($-\log_{10}$(p-value) in expression. In the RC-SC comparison, 313 of the

1,786 down-regulated genes were classified in this category and ranged from 6.94 to 1.30 ($-\log_{10}$(p-value) in expression. For the SI-SC comparison, 145 of the 900 genes that were down-regulated in this catergory ranged from 4.98 to 1.30 ($-\log_{10}$(p-value) in expression. Eighty-two of the 386 genes down-regulated in the RI-RC comparison demonstared expression values ranging from 3.37 to 1.30 ($-\log_{10}$(p-value) and were annotated as genes associated with tanscription.

8. Plant glycolysis

The genes that demonstarted <1.5 fold differential expression in the four comparisons and were involved in plant glycolysis pathway were grouped under this category (Table 2.6) which included; 78 of the 1,965 genes from the RI-SI comparison with expression values ranging from 6.15 to 1.30 ($-\log_{10}$(p-value), 54 of the 1,786 from RC-SC comparison with expression values ranging from 7.00 to 1.30($-\log_{10}$(p-value), 31 of the 900 genes from the SI-SC comparison with expression values ranging from 4.78 to 1.31 ($-\log_{10}$(p-value) and only 14 of 386 genes from the RI-RC comparison with expression values ranging from 2.31 to 1.31 ($-\log_{10}$(p-value).

9. Cell function overview

Genes associated with cell division, cell cycle, development, cell organization, protein modification, protein synthesis, DNA synthesis, DNA repair, unclassified or no ontology and vesicular transport were grouped under the cell function overview category (Table 2.6). This category mainly included genes that were down-regulated and consisted of; 449 of the 1,965 down-regulated genes from the RI-SI comparison with expression values ranging from 5.79 to 1.30 ($-\log_{10}$(p-value), 396 of the 1,786 genes from the RC-SC comparison with expression values ranging from 5.61 to 1.30 ($-\log_{10}$(p-value), 167 of the 900

genes from the SI-SC comparison with expression values ranging from 4.67 to 1.31 (-$\log_{10}$(p-value) and 44 of the 386 genes from the RI-RC comparison with expression values ranging from 3.49 to 1.30 (-$\log_{10}$(p-value).

10. Hormones

Genes with functions involved in hormone biosynthesis and/or hormone signaling were grouped in the horomones category (Table 2.6). This category mainly consisted of genes that were down-regulated. In the RI-SI comparison, 71 of the 1,965 down-regulated genes were grouped in the hormone category. These 71 genes had expression values ranging from 5.61 to 1.30 (-$\log_{10}$(p-value). In the RC-SC comparison, 97 of the 1,786 down-regulated genes were annotated with functions associated with hormones biosynthesis and/or hormone signaling and these genes demonstarted expression values ranging from 6.21 to 1.30 (-$\log_{10}$(p-value). Similarly, 38 of the 900 genes from the SI-SC comparison ranged from 3.45 to 1.30 (-$\log_{10}$(p-value) in expression in this category. A comparison of the RI-RC treatments indicated that 22 of the 386 down-regulated genes were associated with hormones biosynthesis and/or hormone signaling. These 22 genes had expression values ranging from 3.03 to 1.32 (-$\log_{10}$(p-value).

Confirmation of differentially expressed genes

Genes selected from defense related category obtained from the microarray were used to perform real-time PCR and confirm the expression of the genes. Primers designed from selected up- and down- regulated genes were used to amplify RNA isolated from maize lines NSL 30060 and PI 511562 that were inoculated with *U. maydis* (RI and SI) and mock inoculated with water (RC and SC) (Table 2.7). Although the extent of expression was slightly different between the microarray and real-time PCR, the direction of expression was

the same. The slight difference in expression is likely attributed to differences in sensitivity between the two methodologies.

Discussion

Microscopic studies have provided comprehensive information regarding the infection and development of *U. maydis* infection in maize genotypes (Doehlemann *et al*., 2008). Currently there is only modest information regarding the molecular events related to susceptible and resistant reactions in response to *U. maydis* infection in maize plants. Global gene expression studies have proven to be useful for identifying genes associated with specific traits. Therefore, differentially expressed genes were analyzed in resistant and susceptible maize lines, in response to *U. maydis* infection to: 1) Better understand defense responses and determine if activation of plant defenses in maize is similar for *U. maydis* and other biotrophic pathogens (Van de Mortel *et al.*2007, Panthee *et al*., 2009).

It has been predicted that the plant recognizes *U. maydis* due to the presence of conserved molecular patterns also known as Pathogen Associated Molecular Patterns (PAMP). These are recognized by PAMP receptors, which mainly include the leucine rich repeat (LRR) receptor kinases and receptor like kinases which lack the extracellular LRR domain. It has been shown that these PAMP receptors are transcriptionally up-regulated after elicitation (Zipfel *et. al*., 2004, 2006). During the initial phase of host colonization there were 5 genes (*Zm.6372.1.s1_at, Zm.8321.1.s1_at, Zm.17311.1.s1_at, Zm.2872.1.s1_at, Zm.3309.1.s1_at*) that were up regulated in the RI-SI comparison which were classified as receptor like kinases, indicating that the plant is capable of recognizing pathogen attack due to PAMPs. Further, Garcia-Brugger *et al*. (Garcia-Brugger *et al*., 2006) reported that genes encoding kinases and MAPKS cascades as well as oxidative stress-associated genes are all

related with the establishment of innate immune responses at particular stages during early pathogen infection processes. A significant increase in the expression of genes associated with protein kinases, receptor-like protein kinase, serine/threonine kinases, and zinc fingers in the resistant and susceptible inoculated comparison (RI-SI) with respect to the mock inoculated treatments (RC-SC), indicate that the resistant plant recognizes the pathogen at the early infection stages and activates downstream signaling cascades leading to plant defense. In addition, the majority of the up-regulated genes in the RI-SI comparison were grouped in the defense related genes category as compared to the remaining three comparisons (RC-SC, SI-SC and RI-RC). There were 106 genes out of a total of 230 genes that were grouped in the defense related genes category indicating that the resistant plant is capable of recognizing the presence of the pathogen and up- regulating these genes in order to overcome pathogen infection.

It has been demonstrated that hormone signaling in plants is significantly altered due to pathogen attack. These changes are different for necrotrophic and biotrophic pathogens. In compatible interactions with necrotrophic pathogens, Jasmonic Acid (JA) signaling plays a minor role, and instead Salicylic Acis (SA)-dependent cell death responses and the expression of a large set of defense genes including PR1 are observed (Seo *et. al.*, 2001). Biotrophic pathogens, on the other hand, induce JA and ethylene responses during compatible interactions. These responses do not lead to cell death and are associated with induction of tryptophan biosynthesis, the accumulation of secondary metabolites and the induction of plant genes encoding defensins (Brader and Orlandi 2001, Glazebrook, 2005, Wasternack, 2007). Similarly, after infection with *U. maydis*, PR1 expression was undetectable indicating that the SA-dependent cell death response was not activated.

Induction of JA signaling which antagonizes the SA pathway (Glazebrrok, 2005) was detected immediately after infection. JA produced during biotrophic interactions inhibits cell death and is associated with induction of tryptophan biosynthesis. There was significant up-regulation of genes associated with amino acid metabolism/synthesis (aromatic aa. tryptophan) (*Zm.10799.1.s1_at*) in the RI-SI comparison indicating that JA is produced during *U. maydis* infection. JA synthesis is not dependent on the expression level of its biosynthetic genes, but on substrate availability of stored precursors (Waternack, 2007). At the same time activation of JA responsive defense genes such as defensins, lipooxygenase and chitinase were observed, indicating that the resistant plant employs the JA signaling pathway in response to the biotrophic pathogen. The expression of hormone metabolism (jasmonate.synthesis-degradation.lipoxygenase) genes (*Zm.3303.1.a1_at*) with higher expression values in the RI-SI comparison with respect to SI-SC comparison indicated that in the resistant plant there is rapid degradation of JA to prevent the biotrophic pathogen from inhibiting cell death. In accordance with this there was no induction of *Zm 13677.1*, a homologue to the OPR7 gene from rice that has been shown to be essential for JA synthesis (Tani *et al*., 2008).

Elevated levels of auxin are seen in *U. maydis* induced tumors (Turian and Hamilton, 1960). It has been shown that the auxin produced by *U. maydis* is unlikely to be important for tumor formation (Reinecke *et al.*, 2008). The differential expression of auxin responsive genes was observed at 24hpi. These genes were up-regulated which is in agreement with the recently seen transcriptional induction of both auxin synthesis and auxin responsive genes during tumor development, suggesting that the cell enlargement observed in *U. maydis* induced tumors is caused by elevated levels of auxin produced by the plant (Doehlemann *et*

al., 2008). It has been reported in *A. thalianai* that salicylic acid (SA) is repressed due to auxin signaling and vice versa (Wang *et al.,* 2007). The repression of auxin signaling due to SA leads to plant resistance and inhibition of SA signaling allows auxin signaling leading to fungal growth and host susceptibility. This is in accordance with the minor expression of a gene (*Zm.18058.1.a1_at*) associated with salicylic acid synthesis-degradation function that was observed in the RI-SI comparison.

Plant cells produce active oxygen during interactions with potential pathogens. Active oxygen species, including superoxide, hydrogen peroxide, and the hydroxyl radical, could potentially affect many cellular processes involved in plant/pathogen interactions. The active oxygen produced in response to pathogens and elicitors has been hypothesized to have direct antimicrobial effects and to play a role in other defense mechanisms including lignin production, lipid peroxidation, phytoalexin production, and the hypersensitive response (Baker and Orlandi, 1995). *U. maydis,* a biotrophic pathogen avoids cell death, however the hypersensitive reaction is one of the resistance mechanisms in plants that limit the pathogens spread by immediately killing infected plant cells. The reactive oxygen species produced during hypersensitive response are cytotoxic if they are not removed. The reactive oxygen species are detoxified by glutathione-S-tranferase (GST) a detoxification enzyme that plays an important role in pathogen-resistance in plants mainly via peroxide and xenobiotic detoxification (Sugiyama and Sekiya, 2005, Luo *et al.,* 2005, Rouhier *et al.,* 2008). Three GST genes (*Zm.558.1.S1_s_at, Zm.545.1.s1_at, Zm.548.1.s1_at*) were induced at 24 hpi with the highest expression in the RI-SI comparison indicating that the resistant plant is capable of inhibiting pathogen growth by producing HR at the site of infection. A gene (*Zm.16272.1.a1_at*) involved in glutathione synthesis (glutathione synthetase and glutatmate-

cysteine ligase) was significantly up-regulated, and the glutathione content was seen to be increased throughout infection. This indicates the need for a higher antioxidative capacity in the infected plant cells. This increased glutathione content serves as a signal for defense gene induction, as has been reported in *A. thaliana* (Senda and Ogawa, 2004). A number of Cytochrome P450 (*Zm.8654.1.a1_at, Zm.8737.1.a1_at, Zm.14226.1.a1_at, Zm.14226.1.a1_at, Zm.14226.1.A1_at*) members were found in the RI-SI and RC-SC comparisons with the higher expression in the later and are expected to have a similar role in plant defense. Furthermore, members of the P450 family have been shown to be involved in isoflavonoid biosynthesis pathway (Akashi *et. al* 1999; Shimada *et. al* 2000). Genes related to isoflavone, carotenoid, glucosinolares, terpene biosynthesis are of generally related to secondary metabolites and plant defense, whereas ABC transporter, P450, GST genes are related to disease resistance and detoxification processes (Halkier and Gershenzon; 2006, Martin et al 2003, Rea 2007,Schuler and Werck- Reischhart 2003, Panthee et. al 2009). Higher expression of these genes in the RI-SI comparison as opposed to the RC-SC indicates that a number of the general defense related genes were detected and are likely related to resistance.

It has been widely reported that genes in the phenylpropanoid synthesis pathway are among the most powerful antioxidants in plant cells, and they are notably up-regulated in soybean in response to challenge by *P. syringae* (Zou *et al*. 2005; Zabala *et al*. 2006). Similarly, van de Mortel *et al.* (2007) have reported that there are at least ten genes involved in phenylpropanoid synthesis in resistant and susceptible genotypes of soybean in response to Asian soybean rust infection (van de Mortel *et al.*). In this study, seven genes (*Zm.7998.1.a1_at, Zm.8578.3.s1_at, Zm.2631.1.s1_at, Zm.11972.1.a1_at Zm.1567.1.s1_at,*

Zm.8188.1.a1_at, Zm.2550.2.a1_at) involved in phenylpropanoid synthesis were differentially expressed in the RI-SI comparison and the SI-SC comparison. The up-regulation of these genes indicates that the resistant plant recognizes the presence of the pathogen and employs a hypersensitive response (HR) to inhibit the pathogen at the site of infection, the antioxidant activity shown by members of the phenylpropanoid pathway are essential to prevent damage to plant cells due to reactive oxygen species produced during the HR. Further, the up-regulation of genes in the multibranched phenylpropanoid pathway leads to accumulation of toxic metabolites consisting of phytoalexins and anti-microbial compounds including pathogenesis-related proteins and cell wall components such as hydroxyproline-rich glycoproteins and lignin precursors that are capable of inhibiting pathogen infection and disease development (Schmelzer *et al*., 1989, Winkel-Shirley.,2001, Zabala *et al.,* 2006).

It has been reported that the three enzymes; cytochrome P450 monooxygenase, lipoxygenase, and α-dioxygenase, play important roles in preventing the potentially harmful effects of free fatty acid accumulation that could be induced by lipases in the early responses of plants against stress and pathogens (Croft *et al.*, 1993, Blee 2002). Evidence for accumulation of fatty acid derivatives in plant–microbe interactions has been widely reported (Blee 2002; La Camera *et al.*, 2004). In this study the expression of, cytochrome P450 monooxygenase (*Zm.8654.1.a1_at Zm.14226.1.a1_at*), lipoxygenase (*Zm.3303.1.a1_at*), and α–dioxygenase (*Zm.62.1.s1_at*) in the RI-SI comparison as compared to the RC-SC comparison, with higher expression in the later, suggest that these enzymes play a critical role in plant defense responses. A number of genes involved in lignin biosynthesis and lignan synthesis were up-regulated in the RI-SI comparison. This induction is likely due to the

increased cell wall synthesis resulting from enhanced cell division and cell expansion within tumor tissue. This is in agreement with the reinforcement of cell walls by phenolics that has been reported to be associated with defense reactions against the attaching pathogen (Bruce and West., 1989, Egea *et al*., 2001, Huang and Hartmanschimdt, 1992).

Pathogenesis-related (PR) genes have been associated with the development of systemic acquired resistance and encode anti-microbial proteins (Soria-Geerra *et al*., 2010). In this study, PR genes were up- regulated in the RI-SI comparison and RC-SC comparison, with higher expression in the former. PR proteins have deleterious activities toward structural components of pathogens; for example, β-1, 3-glucanases and chitinases (PR-3; Chitinase A, PR-4; Chitinase D) attack fungal cell walls (Fritig *et al*,. 1998). In this study β-1, 3-glucanases (*Zm.6450.1.s1_at*) was seen only in the RI-SI comparison; whereas, the chitinase (*Zm.2227.1.A1_at, Zm.16805.3.S1_a_at, Zm.16805.8.S1_at, Zm.11654.1.A1_at, Zm.847.1.S1_at*) gene was seen in the RI-SI comparison as well in the RC-SC with higher expression in the former. The chitinases genes were also reported by Doehlemann *et al.,* in 2008 indicating that they are essential for the plant and play an important role in overcoming pathogen infection. PR-5 also known as thaumatin like proteins which include the osmotins, are associated with antifungal activity against a range of pathogenic and nonpathogenic fungi (Roberts *et al.,* 1990, Abad *et al.,* 1996). The maize PR-5 like protein also known as Zeamatin was up-regulated in the RI-SI comparison (*Zm.8726.1.a1_at, Zm.281.1.S1_s_at, Zm.6659.1.a1_at*) and RC-SC comparison with higher expression in the former. This indicates that the resistant plant recognizes the pathogen and expresses PR (PR-5) genes that have antifungal activity to overcome the infection. In addition to these PR genes, there was significant expression of the PR-7 (subtilase/PR-P69) gene (*Zm.18432.1.S1_at*) observed in

the RI-SI comparison and RC-SC comparison with higher expression in the former. The PR-7 gene encodes a serine protease that is associated with plant defense. Serine proteases can be induced after a pathogen attack leading to hypersensitive response (HR)-a complex, early defense process, which causes necrosis and cell death in order to restrict growth of the pathogen. The expression of subtilase (PR-P69) was studied by Tornero *et al.*, in tomato plants infected with viroids, confirming that PR-7 is expressed in response to pathogen attack and is associated with plant defense.

A comparison of the up-regulated genes observed in at least three comparisons (RI-SI, RC-SC, SI-SC) demonstrated that there were three genes that that had the highest expression in the RI-SI comparison and were also observed in the RC-SC comparison and SI-SC comparison. These genes are classified as signaling receptor kinases, cell wall modification genes and chitinases.

Phenotype scoring of the maize plants indicated that ther was an accumulation of anthocyanin pigmentation. Additionally, there was an up-regulation of a gene (*Zm.62.1.s1_at*) that encodes for anthocyanin biosynthesis enzyme (secondary metabolite) in the RI-SIcomparison. Anthocyanin accumulation is a part of the response towards a variety of biotic and abiotic stress situations such as pathogen attack, waterlogging, high light, salinity or cold stress (Chalker-Scott, 1999). Since *U. maydis* is a biotrophic pathogen, it does not have direct contact with the anthocyanins in the vacuole. It is likely that the phenotype response and the accumulation of anthocyanin genes is an indirect stress response caused by the fungus.

It is important to point out that the majority of differentially expressed genes identified in this study have unknown functions. Therefore, this large pool of genes should be

investigated further as they may also play specific roles in resistance against *U. maydis*. In conclusion, in this study, a comprehensive list of transcripts regulated in the RI-SI and the RC-SC experimental trials at 24 hpi has been identified. A distinct difference between the resistant and susceptible inoculated (RI-SI) and mock (RC-SC) inoculated comparisons was seen. These findings provide new insights into the complex changes in plant gene expression occurring globally in response to *U. maydis*. Some of these genes can serve as potential targets for genetic improvement of maize plants for enhanced resistance.

There were a significant number of genes that were down-regulated and were classified into different categories based on their function. However, it is difficult to correlate the down-regulation of these genes to be associated directly/indirectly with plant defense. This is also supported with the limited information available to explain the down-regulation of genes during a biotrophic interaction. However, *U. maydis* is a biotrophic pathogen that requires a constant food supply for its growth and development. The resistant plant shows a significant up-regulation of genes associated with plant defense function to overcome the infection. In addition, it also down-regulates genes involved in photosynthesis and plant glycolysis to deplete nutrients to the biotrophic pathogen *U. maydis.* There were 50 genes from the photosynthesis category and 78 genes from the plant glycolysis category that were down-regulated and present in the RI-SI comparison. Thus it is clearly seen that a significant number of genes grouped in the photosynthesis and plant glycolysis category were down-regulated in the RI-SI comparison with respect to other three comparisons.

In conclusion in this study, we have identified a comprehensive list of transcripts that showed a distinct difference between resistant and susceptible *Zea mays* genotypes following *U. maydis* infection. These findings provide insight into the complexity of biotrophic

interactions in an incompatible interaction and indicate that the activation of plant defenses in response to *U. maydis* infection is similar to other biotrophic interactions

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Table 2.1 Total number of differentially expressed genes identified in the four different comparisons at 24 hours post inoculation.

Comparisons	Number of up-regulated genes	Number of down-regulated genes
RI[a]-SI[b]	529	5110
RC[c]-SC[d]	532	4872
RI[a]-RC[c]	2	1160
SI[b]-SC[d]	86	2319

[a] RI- Resistant (NSL 30060) Inoculated (*U. maydis* inoculated)
[b] SI- Susceptible (PI 511562) Inoculated (*U. maydis* inoculated)
[c] RC- Resistant (NSL 30060) Mock Inoculated (water)
[d] SC- Susceptible (PI 511562) Inoculated (water)p-value < 0.05 was applied.

Table 2.2 Number of genes classified into different categories for the up and down-regulated genes observed in the four different comparisons.

Comparison	Number of up-regulated genes	Number of down-regulated genes
RI[a]-SI[b]	230	1965
RC[c]-SC[d]	229	1787
RI[a]-RC[c]	2	386
SI[b]-SC[d]	51	900

[a] RI- Resistant (NSL 30060) Inoculated (*U. maydis* inoculated)
[b] SI- Susceptible (PI 511562) Inoculated (*U. maydis* inoculated)
[c] RC- Resistant (NSL 30060) Mock Inoculated (water)
[d] SC- Susceptible (PI 511562) Inoculated
p-value < 0.05 was applied.

Table 2.3 Classification of up-regulated (≥1.5 fold change) genes for the four comparisons into different pathways.

Pathway	RI[a]-SI[b]	RC[c]-SC[d]	SI[b]-SC[d]	RI[a]-RC[c]
Plant Defense	106	96	31	2
Enzyme families	22	30	4	
Metabolism overview	35	47	10	
Photosynthesis	5	6	2	
Receptor-like-kinase	5	9	1	
Regulation overview	26	15	1	
Transcription	31	26	2	
Total	230	229	51	2

[a] RI- Resistant (NSL 30060) Inoculated (*U. maydis* inoculated)
[b] SI- Susceptible (PI 511562) Inoculated (*U. maydis* inoculated)
[c] RC- Resistant (NSL 30060) Mock Inoculated (water)
[d] SC- Susceptible (PI 511562) Inoculated

Table 2.4 Classification of down-regulated genes for the four comparisons in to different pathways.

Category	RI[a]-SI[b]	RC[c]-SC[d]	SI[b]-SC[d]	RI[a]-RC[c]
Biotic stress	12	38	5	11
Cell function	449	396	167	44
Enzyme families	37	256	270	75
Hormones	71	97	38	22
Metabolism	728	449	202	105
Photosynthesis	50	40	10	7
Plant glycolysis	78	54	31	14
Receptor-like-kinase	138	73	16	27
Regulation overview	49	70	5	10
Transcription	353	313	145	82
Total	1935	1786	900	386

[a] RI- Resistant (NSL 30060) Inoculated (*U. maydis* inoculated)
[b] SI- Susceptible (PI 511562) Inoculated (*U. maydis* inoculated)
[c] RC- Resistant (NSL 30060) Mock Inoculated (water)
[d] SC- Susceptible (PI 511562) Inoculated

Table 2.5 Range of expression values obsereved for the up-regulated genes classified in different categories.

Category	RI[a]-SI[b] ($-\log_{10}$(p-value)	RC[c]-SC[d] ($-\log_{10}$(p-value)	SI[b]-SC[d] ($-\log_{10}$(p-value)	RI[a]-RC[c] ($-\log_{10}$(p-value)
Biotic stress	9.05 – 1.52	9.18 – 1.37	6.40 – 1.33	2.64 – 1.85
Enzyme families	9.84 – 1.54	9.64 – 1.40	5.34 – 1.38	
Metabolism	7.90 – 1.48	10.37 – 1.39	6.36 – 3.37	
Photosynthesis	7.72 – 2.18	8.37 – 1.51	1.97 – 1.90	
Receptor-like-kinase	6.23 – 1.34	6.90 – 1.42	3.86	
Regulation overview	6.59 – 1.31	5.95 – 1.50	2.74	
Transcription	8.98 – 1.61	9.50 – 1.56	3.96 - 2.30	

[a] RI- Resistant (NSL 30060) Inoculated (*U. maydis* inoculated)
[b] SI- Susceptible (PI 511562) Inoculated (*U. maydis* inoculated)
[c] RC- Resistant (NSL 30060) Mock Inoculated (water)
[d] SC- Susceptible (PI 511562) Inoculated

Table 2.6 Range of expression values observed for the down-regulated genes classified in different categories.

Category	RI[a]-SI[b] ($-\log_{10}$(p-value)	RC[c]-SC[d]($-\log_{10}$(p-value)	SI[b]-SC[d] ($-\log_{10}$(p-value)	RI[a]-RC[c] ($-\log_{10}$(p-value)
Plant Defense	5.00 – 3.48	4.53 – 1.31	3.59 – 2.56	3.46 – 1.36
Cell function	5.79 – 1.30	5.61 – 1.30	4.67 – 1.31	3.49 – 1.30
Enzyme families	5.41 – 2.10	5.42 – 1.30	4.28 – 1.37	3.83 – 1.30
Hormones	5.61 – 1.30	6.21 – 1.30	3.45 – 1.30	3.03 – 1.32
Metabolism	6.57 – 1.30	6.34 – 1.30	4.94 – 1.31	4.08 – 1.30
Photosynthesis	4.27 – 1.30	4.80 – 1.37	3.10 – 1.31	2.79 – 1.33
Plant glycolysis	6.15 – 1.30	7.00 – 1.30	4.78 – 1.31	2.31 – 1.31
Receptor-like-kinase	7.37 – 1.30	4.85 – 1.31	4.30 – 1.30	2.94 – 1.30
Regulation overview	4.32 – 1.36	7.40 – 1.31	4.25 – 2.67	3.34 – 1.30
Transcription	7.25 – 1.30	6.94 – 1.304	4.98 – 1.30	3.37 – 1.30

[a] RI- Resistant (NSL 30060) Inoculated (*U. maydis* inoculated)
[b] SI- Susceptible (PI 511562) Inoculated (*U. maydis* inoculated)
[c] RC- Resistant (NSL 30060) Mock Inoculated (water)
[d] SC- Susceptible (PI 511562) Inoculated

Table 2.7 Primers used for quantitative RT-PCR.

Primer Name	Affymetrix Probe ID	Primer Sequence
Zm.8726_F	*Zm.8726.1.a1_at*	5'GCTAGGAATAGCATCCATGGCGT-3'
Zm.8726_R	*Zm.8726.1.a1_at*	5'GCATTGCATATTGCATCGTCGTC3'
Zm.15280_F	*Zm.15280.1.A1_s_at*	5'ACAATGGCACCGAGGCTA3'
Zm.15280_R	*Zm.15280.1.A1_s_at*	5'TCGTGGTCGTAGTACTGC3'
Zm.3303_F	*Zm.3303.1.a1_at*	5'ATCGAGAAACGCGTCGTCA3'
Zm.3303_R	*Zm.3303.1.a1_at*	5'ACAATACCGCGCAAGTGAG3'
BML-28-F	*Zm.686.1.S1_at*	5'CACAAGCCTGAGCTAGT3'
BML-447- R	*Zm.686.1.S1_at*	5'TCGTCGACGGTGACGATCTCGTCAG 3'
KNA-25-F	*Zm.10830.1.s1_at*	5'ATCGTTTGGCAATCTGACT 3'
KNA-479-R	*Zm.10830.1.s1_at*	5'CATATGGCGCATGACGCA T 3'

Figure 2.1 *Zea mays* NSL 30060 demonstrating a disease rating score ranging from 1-2. a. The minor leaf chlorosis exhibited by white streaks on the leaves. The phenotype corresponds to a. 1C resistance reaction rating score. b. Anthocyanin production exhibited by the purple leaf color. The phenotype corresponds to a 1A resistance reaction rating score. c. Minor leaf gall development that corresponds to a 2 resistance reaction rating score.

a. 

b. 

c. 

Figure 2.2 Maize lines (Ames 22443, Ames 27104 and PI 511562) demonstrating a disease rating score ranging from 3-5. a. Susceptible maize plants with severe stem gall development and black teliospores. The phenotype corresponds to a 3 resistance reaction rating score. b. Susceptible maize plants with severe basal gall development. The phenotype corresponds to a 4 resistance reaction rating score.

a. 

b. 

CHAPTER 3

RESISTANCE TO *USTILAGO MAYDIS* IN MAIZE, TEOSINTE AND TEOSINTE INTROGRESSION LINES (NILS)[1]

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Abstract

Wild ancestors of crop plants have been used for providing pest and/or disease resistance or abiotic stress tolerance for use in producing hybrids, or improving quality traits of the cultivated crops. Progenitor species have provided plant breeders with a broad pool of potentially useful genetic resources and therefore have been used to improve modern agriculture. Teosinte, the wild progenitor of maize, has demonstrated resistance to various diseases and insects over an extended period of time. However, there have been limited reports of the use of teosinte to improve the resistance of maize against *Ustilago maydis*,, the causal agent of corn smut disease. The significant economic loss incurred due to *U. maydis* (1.24 billion in 2013) in maize and the lack of resistant maize cultivars necessitates the identification of new sources for resistance. We inoculated maize, teosinte and maize-teosinte introgression lines (NILs) with a strain of *U. maydis* and identified one maize line, two teosinte lines (*Zea diploperennis* and *Zea luxurians*) and two maize-teosinte NILs (Z031E0068, Z031E1068) with a high level of resistance to *U. maydis*. The teosinte introgressed region present in the NILs is 3.9 Mbp in size and carries 7 genes with functions associated with plants defenses. These genes were therefore characterized as candidate genes that are potentially contributing to the resistant phenotype. This is the first report of the identification of new sources of resistance to *U. maydis* from maize, teosinte and maize-teosinte NILs.

Introduction

Globalization of agriculture has resulted in the growth of crop plants having a narrow genetic base in areas far away from their centers of origin and therefore far from the pathogens that have co-evolved with them. Therefore these plants are unlikely to have evolved resistance to new strains of the pathogen that may have subsequently arisen in the center of origin (Strange and Scott 2005). In addition to this the crops introduced to a new area may be poorly equipped to resist pathogenic organisms that may be resident there. Plants are subject to disease both in the field and post-harvest, the major group of pathogens being fungi, bacteria, viruses, oomycetes, nematodes and parasitic plants. Solutions to the problems of plant disease include quarantine, exclusion, rotation, intercropping and chemical control. However the most effective method is host resistance, which can be accomplished by exploiting the gene pool of the plant and its relatives in breeding programs.

Plant breeding involves genetic improvement of crops to produce new varieties that have increased productivity and quality. This can be achieved by breeding resistance genes from a resistant cultivar into a susceptible cultivar by making controlled crosses and screening the progeny lines for resistance reaction. These progeny lines are then validated for the resistant phenotype by using molecular markers or gene expression analysis. The traditional method of breeding plant for resistance is to cross the exotic germplasm (containing the resistance gene) with the cultivated species (lacking the resistance gene). This method is effective when the trait of interest is controlled by one or a few genes. The progeny lines are then screened for the resistance phenotype. This involves inoculating hundreds or thousands of plants with a given a pathogen. One or more resistance lines are identified and used to make further crossing experiments. This methodology is known as marker assisted

selection and is the most effective way of pyramiding multiple genes in a single cultivar. Marker assisted selection is not only cost-effective and efficient; it is also amenable to automation and high throughput. It can be applied for the maintenance of recessive alleles in backcrossing pedigrees and for pyramiding of resistance genes (Hajjar and Hodgkin 2007).

The centers of origin of plants consist of wild ancestral species that have the greatest genetic diversity and have co-evolved with pathogens. This has resulted in the identification of resistant germplasm by plant breeders with the aim of introgressing the resistance genes for agricultural benefits (Leppik 1970). Wild progenitor species have been used to improve cultivated crop performance since 1920s, wherein they served as a source of plant genetic resource (Loskutov 1999). However in the 1940-1950 agricultural researchers began using them to improve major crops (Plucknett *et al.*, 1987, Hodgkin *et al.* 1992). Later in the 1960-1970, plant breeders were successfully able to use wild progenitors in breeding programs to improve cultivated varieties (Harlan 1976, 1984; Hawkes 1977; Prescott-Allen and Prescott-Allen 1986; Hoyt 1988). In the following years 1980-1990, genetic engineering tools allowed crop improvement by utilizing genes from distantly related taxa to be incorporated into cultivated crops thereby broadening the value of wild progenitor species (Meilleur *et al.*, 2003). The progenitor species provide plant breeders with a broad pool of potentially useful genetic resources and therefore have been used to improve modern agriculture (Hajjar and Hodgkin 2007). This is beneficial since cultivated species often lack genes required by the plant breeders, particularly genes for disease resistance. In addition to this, in a number of crops the known genes for disease resistance are being used up as they are released in cultivars and then overcome by new races of pathogens. Thus wild ancestors of crop plants are being used as a source of genetic variation to develop higher yielding, nutritious and

environmentally friendly varieties that can improve our quality of life without harnessing additional natural habitats to agricultural production (Zamir 2001).

The ancestral species have been used for providing pest and/or disease resistance or abiotic stress tolerance, increasing yield, providing cytoplasmic male sterility or fertility restoration for use in producing hybrids, or improving quality traits of the cultivated crops. However the most common use of wild species is as a source of pest or disease resistance. The use of tomato wild progenitors to improve resistance has been reported at a rate of one per year since 1982 (Rick and Chetelat 1995) and over 40 resistance genes in cultivated tomato are from *Lycopersicon peruvianum, L. cheesmanii, L. pennellii* and several other wild relatives (Rick and Chetelat 1995). Other examples of wild species used to improve resistance are seen in rice, potatoes, wheat, cassava, banana, sorghum and lettuce. To elaborate, in rice the wild progenitor *Oryza nivara* has been used to improve resistance against grassy stunt virus and in cultivated potato, wild progenitors *Solanum demissum and S. stoloniferum* have been used to incorporate potato late blight resistance. Further, resistance in cultivated wheat varieties against leaf rust, stem rust, yellow dwarf virus, root lesion nematode, powdery mildew and wheat streak mosaic virus has been incorporated from the wild *Aegilops tauschii* (Hoisington *et al.,* 1999, Suszkiw 2005, Hajjar and Hodgkin 2007). The rapid increase in use of wild progenitors to improve resistance is seen with the increasing number of cultivated crops that comprise genes from their ancestral species. This is also seen in sunflower which contains multiple sources of genetic resistance to all known races of downy mildew, rust, verticillum wilt, broomrape and herbicide resistance obtained from wild *Helianthus annuus* and *H. praecox* (Hajjar and Hodgkin 2007). In addition to the above stated examples there are numerous other examples like *Mycosphaerella fijiensis*

resistance in banana obtained from wild *Mus acuminata,* and resistance to soybean cyst nematode in soybean successfully transferred from wild perennial soybean, *Glycine tomentella* (Wilson *et al.,* 1991, Wilson and Gates 1993, Escalant *et al.,* 2002, Riggs *et al.,* 1998, Hajjar and Hodgkin 2007). These examples clearly indicate that the use of wild relatives has continued to increase in terms of the number of crops with cultivars containing genes from wild relatives. The number of wild species used to provide genes, and the range of traits obtained from wild relatives has also increased significantly over the past decade (Prescott-Allen 1986, 1988, Hajjar and Hodgkin 2007). However the use of wild genes in groundnut and maize has not been as successful so far. The possible explanation for this is retention of undesirable agronomic traits and poor agronomic performance incorporated from the wild progenitors. However these deleterious effects of cross breeding with wild relatives can be overcome through backcrossing and use of molecular techniques to screen the progeny lines for resistance reaction.

Teosinte is the common name for the wild taxa of *Zea* and is the progenitor of Maize. The genus *Zea* is further divided into two sections namely; *Luxuriantes* and *Zea. Luxuriantes* comprises of three species; *Z. diploperennis* Z. *perennis* and *Z. luxurians*. Four subspecies constitute the section *Zea,* these are; *Zea mays* L. ssp. *huehuetenangensis, Zea mays* L. ssp. *parviglumis, Zea mays* L. ssp. *mexicana* and *Zea mays* L. ssp. *mays,*). *Zea mays* L. ssp. *parviglumis* is the immediate progenitor of maize. In the seedling growth stage, maize and teosinte look alike however the morphological differences are significant at maturity especially in the inflorescence. Maize consists of a single stalk terminating in a tassel and has two to five ears. The ears of maize consist of numerous naked yellow to white colored kernels arranged in multiple rows on a single cob. On the contrary, teosinte is highly

branched with each branch terminating in long tassel and cobs comprise 5-10 kernels each enclosed in a hard fruitcase (Matsuko *et al.,* 2002). Teosinte has a brittle cob, while maize forms solid that donot release their seeds.

Maize is an economically important crop in the United States, generating approximately 80 billion dollars in the year 2012 (USDA, WASDE, Jan. 11, 2013). In addition to domestic consumption, the United States was the largest maize exporter in the world in 2011. Since maize plays a significant role in current biofuel production, the importance of maize in US agriculture is only expected to increase. A number of biotic and abiotic factors have made maize production more challenging. At present, one of the threats to this crop is corn smut caused by *Ustilago maydis* (*U. maydis*), a Basidomycota fungi. Currently there are no maize cultivars that show complete resistance against *U. maydis*, and no single gene that confers resistance (Smith, J.T., 2011). The partially resistant cultivars are capable of maintaining yield losses limited to 2% (Allen, A., *et. al*. 2011). However, even a 2% loss of 80 million dollars is equivalent to 1.6 million dollars annually which is a significant yield loss. To control corn smut disease, several methods have been recommended, including crop rotation, sanitation, seed treatments, application of foliar fungicides, modification of fertility, and biological controls. However host resistance is the only practical method of managing common smut in areas where *U. maydis* is prevalent (Smith, J. T., 2011). The wild progenitor teosinte can potentially be used to improve resistance of cultivated maize varieties since teosinte crosses readily with maize to produce fertile hybrids. Currently there has been limited information available regarding resistance in teosinte against fungal pathogens. However there have been reports indicating that teosinte is susceptible to some fungi that are pathogenic to maize, but the potential of teosinte species as

a source of resistance against *U. maydis* is unknown (White 1999). Since teosinte is a perennial and grows in sub-tropical environments that lack winter freeze to keep the pathogen populations under control, it must be capable of tolerating significant disease and insect exposure due to its evolutionary histroy span during which it has been exposed to numerous pathogens. Thus our hypothesis is that teosinte can likely be used as a source for improving resistance in modern, annual temperate maize against *U. maydis*.

In order to test our hypothesis, we have inoculated and phenotyped 100 maize-teosinte introgression (NILs) lines with the aim of identifying new sources of resistance against *U. maydis*. One-hundred maize-teosinte introgression lines (NILs) were provided for this work by Dr. Sherry Flint-Garcia at the University of Missouri, Division of Plant Sciences, and USDA-ARS. These lines were created by backcrossing 10 different teosinte (*ssp. parviglumis*) accessions into the maize *B73* background, creating 900 teosinte introgression lines. Each line is near-isogenic and has an average of 4% teosinte from random parts of the genome. The 900 NILs have been genotyped with ~768 SNPs each, therefore regions that have been introgressed into each line are known. A "minimum tiling path" of 100 lines from one teosinte accession was created. The advantage of using NILs is that they are genetic stocks that are nearly genetically identical, differing only at one (or a few) chromosomal segments. The characteristic feature of these NILs population is that each NIL carries a distinct chromosomal segment and when ordered through molecular markers analysis, the final set of NILs represents a "tiling path" of introgression that covers the entire genome. These NILs serve as useful genetic stocks for investigating gene function and regulation since the genetic background effect has been removed. Therefore they allow the introgressed teosinte genome that is potentially contributing to the resistant phenotype to be

precisely analyzed. The uniform genetic backgrounds of the NILs will allow the causal introgressions to be identified and characterized. Based on these considerations, the objectives of this study were to: 1) Identify and characterize new sources of resistance from teosinte and maize-teosinte introgression near isogenic lines (NILs) in response to pathogen (*U. maydis*) infection.

Material and Methods

Plant material

One hundred maize-teosinte introgression lines (NILs) were provided for this work by Dr. Sherry Flint-Garcia at the University of Missouri, Division of Plant Sciences, and USDA-ARS. These lines were created by backcrossing ten different teosinte (*ssp. parviglumis*) accessions into the maize *B73* background, creating 900 teosinte introgression lines. Each line is near-isogenic and has an average of 4% teosinte from random parts of the genome. The 900 NILs have been genotyped with ~768 SNPs each, therefore regions that have been introgressed into each line are known. A "minimum tiling path" of one hundred lines from one teosinte accession was created. The one hundred lines used for this work contain almost the entire teosinte genome with each introgression line carrying a different region from the teosinte genome. These one hundred maize-teosinte intorgression lines were used for *U. maydis* inoculations. In addition to these NILs, three teosinte subspecies namely; *Zea mays parviglumis, Zea mays luxurians* and *Zea mays diploperennis* were also used to study their response on *U. maydis* inoculation. Experimental units were single seedlings with six seeds for each line. Plants were grown in a growth chamber with day and night environments of 28/20°C temperature and 12/12 h of photoperiod, respectively and

approximately 500 μmol m^{-2} sec^{-1} photosynthetically active radiations at the top of the canopy. The relative humidity was maintained during the day and night at approximately 70% and 90%, respectively. All plants were kept in the same growth chamber to maintain a growth environment that is congruent across the experiment.

Ustilago maydis Inoculation

Wild type *U. maydis* strain ½ (mating type a1b1) and a near isogenic strain 2/9 (mating type a2b2) were used for inoculations. Both strains were grown separately in potato dextrose broth at 30^0C to an OD_{600} of 1.0 (~ 1 X 10^7 cells/ml). Cells were suspended in water to a final concentration of 1 X 10^6 cells/ml. Ten days after planting, the culm of the plants were injected just above the soil line with ~100ul cell suspensions containing 1 x 10^6 cells/ml of each of the wild type *U. maydis* strains. The control plants were mock inoculated with water only. After inoculation, the plants were returned to the growth chamber until phenotypic scoring. The seedlings were then scored each day for 21 days beginning 7 days post inoculation (dpi) to monitor the disease progress and identify resistant lines (Chavan, and Smith In press). Phenotypic scoring was done using a disease rating scale of 0 to 5. A standard disease rating system for *U. maydis* was utilized to score the plants with a 0 (highly resistant) to 5 (highly susceptible) infection type rating scale. Three independent experiments were performed consisting of six seedlings for each line.

Results

Phenotype scoring

One hundred maize-teosinte introgression lines (NILs) and three teosinte lines were scored each day for 21 days beginning 7 days post inoculation (dpi) to monitor the disease

progress and identify resistant lines. The disease scoring scale ranged from 0 to 5. Where 0 is healthy (complete resistance) and 5 indicates plant death (complete susceptibility). Intermediate scores include 1C (chlorosis), 1A (anthocyanin pigmentation), 2 (leaf galls), 3 (stem galls) and 4 (basal galls). After inoculation the seedlings were phenotypically scored each day for 21 days beginning 7 dpi to monitor the progress of disease. Phenotypic scoring of the three biological replications identified two maize-teosinte introgression lines, Z031E0068 and Z031E1068 and two teosinte lines *Zea mays luxurians* and *Zea mays diploperennis* that exhibited high levels of resistance to *U. maydis* (Table 3.1). These resistant lines showed symptoms of chlorosis (1C), anthocyanin (1A) and minute leaf galls (2). The minute leaf galls were not clearly visible and could be felt only by touching the leaf blade. These minute leaf galls either disappeared or did not enlarge in size as the plant grew. We therefore considered these plants as resistant. However the remaining ninety-eight maize teosinte introgression lines, including the parents for this population had a disease scoring scale of 3-5. These plants initially showed enormous leaf, stem and basal galls. The plants showing leaf galls later developed stem or/and basal galls. Some plants that showed stem or basal galls died as the disease progressed and thus these plants were considered as susceptible.

Teosinte introgressed region in resistant NILs

The two NILs (Z031E0068 and Z031E1068) that showed a high level of resistance against *U. maydis* had a segment of tesointe introgressed on chromosome 9. Genotype data indicated that Z031E0068 has introgression only on chromosome 9 which is heterozygous having 'AB' genotype (AA-homozygous B73 (Parent 1) and BB-Homozygous *Zea parviglumis* (Parent 2)) and is 3.6 Mbp in size. The second NIL, Z031E1068 is a further

selfed (BC4S4) line of Z031E0068 and has 33,000 SNPs that have been genotyped using RAD markers. This line (Z031E1068) has introgression in four chromosomes, namely chromosome 1, 4, 5, and 9. The introgression size for these chromosomal regions is; chromosome 1 (3.4Mbp), chromosome 2 (1.2Mbp), chromosome 5 (2.1Mbp) and chromosome 9 (3.9Mbp) respectively. In order to narrow down the region from Z031E1068 that is associated with resistance, additional lines containing overlapping introgression in chromosome 1, 4, 5 and 9 were phenotyped. The phenotype data indicated that lines containing introgression in chromosome 1, 4 and 5 were completely susceptible. Concluding that introgression in chromosome 9 from Z031E1068 is likely responsible for the resistant phenotype. Thus, the two resistant NILs Z031E0068 and Z031E1068 have an introgression on chromosome 9 which is 3.9Mbp and 3.6Mbp respectively. The parental lines (B73 – Parent1 and *Zea parviglumis* -Parent 2) for these NILs are completely susceptible to *U. maydis* infection; however two NILs (Z031E0068 and Z031E1068) show a high level of resistance against *U. maydis.* The most plausible explanation of this is the combination of maize and teosinte genes present in the maize-teosinte introgression lines (NILs) that is potentially responsible for the resistant phenotype. This is supported by the fact that resistance against *U. maydis* is quantitatively controlled (multiple genes) (Baumgarten *et al*., 2007). The two resistant NILs having introgression in chromosome 9 were further analyzed to identify genes that are present in teosinte introgressed region and are potentially contributing to the resistant phenotype. Due to a lack of sequence information for the teosinte line, sequence information from B73 (Parent 1) was used. Analysis of the B73 genome sequence indicated that there are a total of 162 genes present on chromosome 9, the region corresponding to the teosinte introgressed region in the resistant NILs (Z031E0068 and

Z031E1068). Out of these 162 genes, 90 genes are uncharacterized and 7 genes have functions associated with resistance. Five genes from a total of 7 genes were receptor like kinases and/or protein kinases, 1 gene had a leucine rich repeat (LRR) domain and 1 gene was a putative subtilase (Table 3.2).

Discussion

Ustilago maydis (*U. maydis*), the causal agent of corn smut is an important agricultural pathogen and is responsible for significant yield losses of approximately $1.24 billion annually in the United States (Allen, A., *et. al*. 2011). Several methods including crop rotation, sanitation, fungicide application, modification of fertility, biological control and seed treatments are currently used to control corn smut. However, host resistance is the only practical method for managing smut. Currently, there are no known maize lines available that show complete resistance against *U. maydis*. The partially resistant lines that are presently employed in agriculture will most likely lose their resistance soon due to the ever evolving pathogen. Since maize is an economically important crop, identification of new and durable sources of resistance is critical. This can be accomplished by breeding resistance genes from a resistant cultivar into a susceptible cultivar. However in a number of crops the known genes for disease resistance are being used up as they are released in cultivars and then overcome by new races of a pathogen. In addition to this cultivated species often lack genes required by the plant breeder particularly genes for disease resistance (Knott 1971). Thus the use of closely related species to introgress resistance genes is being used as an alternative strategy to improve resistance of cultivated crops.

The use of wild progenitors to improve resistance has been successful in many cereal crops, however there are limited reports seen in maize. Further there are no reports indicating

the identification of resistance against *U. maydis* in the wild progenitor teosinte. To fill this gap of knowledge we have identified two new sources of resistance against *U. maydis* from the wild progenitor teosinte namely; *Zea mays luxurians* and *Zea mays diploperennis*. This is the first report of identifying potential new sources of resistance to *U. maydis* from the wild progenitor teosinte. These teosinte lines have the potential to be employed in breeding programs to improve the resistance of maize against *U. maydis*. The use of wild progenitor species to improve resistance has been done previously. Few examples of wild genes preventing devastation by pests and disease were seen, these include *Oryza nivara* providing resistance to grassy stunt virus in rice, *Solanum demissum* providing resistance to potato late blight, *Agropyron elongatum* and *Aegilops umbellulata* providing resistance to stem and leaf rust in wheat. In addition to these there are many tomato disease resistance genes introgressed from wild species, mostly from *Lycopersicon pimpinellifolium* (Prescott-Allen and Prescott-Allen 1986).Thus the use of wild progenitor teosinte to improve resistance of maize against *U. maydis* is possible.

Reports indicate that teosinte and maize are sexually compatible and can be hybridizated to produce progeny lines. However there have been no reports of producing maize teosinte introgression lines with the aim of improving resistance against *U. maydis*. This has been the first report where we have identified two maize teosinte introgression lines (Z031E0068 and Z031E1068) that show high level of resistance against *U. maydis* when compared with the recurrent parent B73. Therefore we have not only identified new sources of resistance from the wild progenitor teosinte, but also shown that these can be used in breeding programs to improve resistance of cultivated maize lines. Secondly, even after making crosses between maize and teosinte we have seen that the resistance against *U.*

maydis is seen in the progeny lines. Therefore the use of teosinte lines as breeding material in the genetic improvement of maize can be extremely useful because it provides durable resistance against *U. maydis.*

The resistant teosinte (*Zea mays luxurians* and *Zea mays diploperennis*) and maize teosinte introgression lines (Z031E0068 and Z031E1068) showed chlorosis and anthocyanin pigmentation with minute to no leaf galls. The production of phenolic compounds like anthocyanin is essential for disease resistance in plants. The accumulation of these phenolic compounds on pathogen infection restricts the pathogen growth by protecting the tissue from accumulation of oxidative metabolites. Prior studies show that there is up-regulation of anthocyanin in maize lines resistant to *Biopolaris maydis,* the causal pathogen of southern leaf blight, while anthocyanins were not found surrounding the lesions of susceptible controls, and the "anthocyanin ring" was only present in healthy tissue where the pathogen had not yet progressed (Hipskid, 1996). Further, there was also no anthocyanin pigmentation present in non-inoculated control plants of either susceptible or resistant varieties. Indicating that anthocyanin is produced only in infected plants and served as a signal for activation of plant defense.

Two maize teosinte introgression lines (NILs) show high level of resistance against *U. maydis* even though both the parents of this population namely; B73 and *Zea mays parviglumis* are susceptible. One of the possible explanations for this is the combination of maize and teosinte genes in the maize teosinte introgression lines that is responsible for the resistant phenotype, since resistance against *U. maydis* is controlled by multiple genes (Baumgarten *et al*., 2007). Another plausible explaination for the resistance seen in the resistant NILs is gene interaction between the maize and teosinte genes that are present in the

two resistant NILs. Gene interaction is most likely contributing to the resistant phenotype. Additionally, suppression of the resistance genes may be another mechanism responsible for the resistant NILs. The presence of suppressors of resistance genes in either parent (B73 or *Zea mays parviglumis*) may inhibit the expression of resistance against *U. maydis* infection. The suppressor gene/genes are most likely absent or mutated in the two resistant NILs resulting in the expression of resistance demonstrated by the resistant phenotype. Suppressors of stem rust resistance genes have been identified in the Canthatch cultivar on chromosome 7DL (Kerber and Aung 1995, 1999) and for the *Lr23* resistance gene on the 2DS chromosome in*Aegilops tauschii* (Nelson *et al.,* 1997). iIt would be really interesting to identify the genes present in the introgressed regions that are potentially contributing to the resistant phenotype. However this requires extensive sequencing and is restricted due to lack of sequence information from the teosinte lines. Therefore to identify the potential genes present in the introgressed regions we have utilized the publically available sequence information of B73 (http://maizegdb.org/) which is the maize parent used to generate the NILs population.

The two resistant NILs Z031E0068 and Z031E1068 have an introgression on chromosome 9 that is 3.9Mbp and 3.6Mbp, respectively. The introgression is most likely contributing to the resistant phenotype. Although Z031E1068 is a further self (BC4S4) of Z031E0068 it has introgressions present in multiple chromosomes that were not identified in the genotype anlaysis of Z031E0068. This can be explained by the number of SNPs identified in the two NILs. The number of SNPs identified in Z031E0068 was ~800 while the number of SNPs identified in Z031E1068 was 33,000. This indicates that the higher number of SNPs in Z031E1068 enabled identification of teosinte introgression in multiple

chromosomal regions that were not observed in Z031E0068. Therefore, by increasing the number of SNP markers in Z031E0068 there is a possibility of identifying multiple introgressions. This will also confirm that the teosinte introgession present in regions other than chromosome 9 are not contributing to the resistant phenotype.

Analysis of the genes present in B73 genome corresponding to the teosinte introgressed region (Chromosome 9) in the resistant NILs, indicate that there are a total of 162 genes present in this region. A majority of these genes are uncharacterized; however we identified 7 genes that have functions associated with plant defense (Table 2). For example, we identified 5 genes that were receptor like kinase and/or protein kinases, 1 gene had a leucine rich repeat (LRR) domain and 1 gene was a putative subtilase. The receptor like kinase and LRR domains are important in plant responses to a variety of external stimuli including pathogens. These are associated with pathogen recognition, leading to activation of plant defense. The LRR region of the classical Nucleotide Binding Site (NBS) LRR proteins serve as receptors that interact directly/indirectly with elicitor molecules for the pathogen. For example in *Arabidopsis* the NBS-LRR resistance genes *RPS2* confers resistance to strains of *Pseudomonas syringae* bacteria that carry the plasmid-borne *avrRpt2* gene (Bent *et al.,* 1996). In addition to this another class of resistance genes known as receptor like kinases are associated with recognition of the pathogen elicitors. This is seen in tomato plants that carry the resistance gene *Pto* and confers resistance to races of *Pseudomonas syringae* pv. *tomato* that carry the *avrPto* gene. The resistance gene *Pto* codes for a 321-amino acid protein and has been shown to be a serine/threonine protein kinase, capable of autophosphorylation (Loh and Martin 1995). This indicates that the receptor kinase and LRR

genes that were identified in the B73 genome are potentially contributing to the resistant phenotype of the two NILs.

Previous studies indicate that subtilase, are pathogenesis-related protein (PR) and were shown to be one of the several subtilases that are specifically induced following pathogen infection in tomato (Vera & Conejero, 1988; Tornero et al., 1996a; Jordá et al., 1999). PR proteins are associated with activation of plant defense mechanism. Indicating that the subtilase identified in the B73 genome corresponding to the teosinte introgressed regions in the resistant NILs is most likely contributing to the resistant phenotype that was seen. The identification of genes associated with pathogen recognition and activation of plant defense indicates that the teosinte region introgressed on chromosome 9 in the two resistant NILs, potentially has genes associated with resistance. These genes are likely contributing to the resistant phenotype. Due to lack of sequence information from teosinte we can only speculate the potential genes that are most likely present in the introgressed region. However there are more genes involved in the resistance reaction and these are most likely from the teosinte genome. It would be interesting to identify the teosinte genes that are present in the introgressed regions by sequencing the teosinte introgressed region present in the resistant NILs. This will generate a list of all the genes present in the introgressed regions in the two resistant NILs. It will also give us a better understanding of the genes that are potentially contributing to the resistant phenotype.

The sequence information of the genes in the teosinte introgressed can be used to design primers and PCR amplify these genes from the two resistant teosinte (*Zea mays diploperennis* and *Zea mays luxurians*) lines. This will enable us to identify and characterize the genes in the resistant teosinte lines that are potentially responsible for the resistance

phenotype. In addition to this the teosinte parent that was used to generate the maize teosinte introgression lines (*Zea mays parviglumis*) is susceptible to *U. maydis* inoculation. It would be feasible to develop a population using the resistant teosinte lines that we have identified in this study (*Zea mays diploperennis* and *Zea mays luxurians*). There is a possibility that we could obtain progeny lines with enhanced resistance against *U. maydis*. These lines can be further phenotyped by performing *U. maydis* inoculation and genotyped using the primers designed from the teosinte introgressed sequence information to evaluate them for resistance against *U. maydis.* This study has given us a starting point to further identify resources for *U. maydis* resistance that are unexploited in teosinte for the breeding of maize cultivars with improved resistance against *U. maydis.*

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Table3.1 Phenotype analysis of teosinte and maize-teosinte introgression lines (NILs).

Lines	1C Chlorosis	1A Anthocyanin	2 leaf gall	3 Stem gall	4 Basal gall	5 Death
Zea mays subsp. luxurians	R	R	R			
Zea mays subsp. diploperennis	R	R	R			
Zea mays subsp. parviglumis				S	S	S
B73 (P1)				S	S	S
Zea mays subsp. parviglumis (P2)				S	S	S
Z031E0068	R	R				
Z031E1068	R	R				

Table 3.2 List of interesting genes present on chromosome 9 of the sequenced B73 genome.

Gene Name	Organism	Chromosome	GO Description	Description
GRMZM2G092776	*Zea mays*	9	protein kinase activity	Putative DUF26 domain receptor-like protein kinase; Receptor protein kinase CRINKLY4 [Source:UniProtKB/ TrEMBL;Acc:B6TWE9]
GRMZM2G175563	*Zea mays*	9	protein kinase activity	Protein kinase domain superfamily protein [Source:UniProtKB/ TrEMBL;Acc:K7VLX5]
GRMZM2G317938	*Zea mays*	9	protein kinase activity	Uncharacterized protein [Source:UniProtKB/ TrEMBL;Acc:C4J566]
GRMZM2G109624	*Zea mays*	9	protein kinase activity	Putative glycogen synthase kinase family protein
GRMZM2G051103	*Zea mays*	9	protein kinase activity	Putative CBL-interacting protein kinase family protein; Uncharacterized protein [Source:UniProtKB/ TrEMBL;Acc:C0P6L2]

Gene Name	Organism	Chromosome	GO Description	Description
AC196090.3_FG006	*Zea mays*	9	serine-type endopeptidase activity	Putative subtilase family protein [Source:UniProtKB/TrEMBL;Acc:K7WHG7]
GRMZM2G172014	*Zea mays*	9	protein kinase activity	Putative leucine-rich repeat transmembrane protein kinase family protein [Source:UniProtKB/TrEMBL;Acc:K7VM01]

CHAPTER 4

CONCLUSIONS

Maize is an economically important crop in United States, generating approximately 62 billion dollars in the year 2012 (USDA, WASDE, Jan. 11, 2013). Apart from domestic consumption, United States has been the largest exporter of maize in 2011 and maize export makes the largest net contribution to the U.S. agricultural trade balance of all the agricultural commodities. The significant role of maize in US agriculture is only expected to increase due to its current use in biofuel production. However a number of biotic and abiotic factors have made maize production more challenging. At present, one of the threats to this crop is corn smut caused by *Ustilago maydis* , a Basidomycota fungi. Currently there are no maize cultivars that show complete resistance against *U. maydis*, and no single gene that confers resistance (Smith, J.T., 2011). The currently available and used partially resistant cultivars are capable of maintaining yield losses up to 2% (Allen, A., *et. al*. 2011). However, even a 2% loss of 62 billion dollars is equivalent to 1.24 billion dollars annually which is a significant yield loss indicating that it is essential to control *U. maydis.* Currently used control methods for corn smut disease, , includecrop rotation, sanitation, seed treatments, application of foliar fungicides, modification of fertility, and biological controls. However host resistance is the only practical method of managing common smut in areas where *U. maydis* is prevalent (Smith, J. T., 2011).

Improving resistance of cultivated maize against *U.* maydis is possible only by identifying new sources of resistance and understanding the plant defense mechanisms against *U. maydis.* This will help to develop strategies for effective control against corn smut disease. Unfortunately, cultivated species often lack genes for disease resistance and known genes for disease resistance are being used up as they are released in cultivars and then overcome by new races of pathogens. In addition to this there is only modest information available about molecular events related to susceptible and resistant reactions on *U. maydis* infection of maize plants. Therefore the overall goal of this project is to identify new sources of resistance against *U. maydis* from the wild progenitor teosinte. More specifically the objectives of this research were to 1) Characterize the expression pattern of differentially expressed genes in maize, and its wild progenitor teosinte in response to pathogen (*U. maydis*) infection by performing a microarray analysis and 2) Identify and characterize new sources of resistance from teosinte and maize-teosinte introgression near isogenic lines (NILs) in response to pathogen (*U. maydis*) infection.

The work performed has identified a maize (*Zea mays subsp. mays* NSL 30060, United States, Wisconsin) line with high level of resistance against *U. maydis*. The study of differentially expressed genes between resistant and susceptible maize lines in response to *U. maydis* infection indicates that the resistant plant recognizes the pathogen attack in the early stages and activates plant defenses. The plant recognizes *U. maydis* due to the presence of conserved molecular patterns also known as Pathogen Associated Molecular Patterns (PAMP) which mainly include the leucine rich repeat (LRR) receptor kinases and receptor like kinases which lack the extracellular LRR domain. This recognition results in activation of downstream signaling cascade leading to plant defense. This is seen due to the up-

regulation of a majority of plant defense genes that were observed in the resistant inoculated maize plants as compared to the susceptible inoculated and control plants. These included the pathogenesis related genes that have anti-microbial/anti fungal activity and are associated with activation of plant defense and development of systemic acquired resistance.

Apart from the plant defense genes there was a significant up-regulation of genes having functions associated with hormone metabolism and synthesis. These mainly included genes involved in Jasmonic Acid (JA) and auxin synthesis and metabolism. The production of JA results in activation of defense genes such as defensins, lipooxygenase and chitinase indicating that the resistant plant employs the JA signaling pathway against the biotrophic pathogen. However JA inhibits cell death and therefore the resistant plant rapidly degrades JA to inhibit the biotrophic pathogen. This is evident by up-regulation of genes involved in JA metabolism pathway that were seen in the resistant inoculated plants.

U. maydis infection is characterized with the formation of galls in all the aerial parts of the plants due to uncontrolled cell division as a result of elevated levels of auxins produced. This occurs due to inhibition of the negative feedback mechanism regulating auxin synthesis pathway. There was significant increase in expression of genes present in the auxin synthesis pathway that was seen in the susceptible inoculated plants. The uncontrolled cell division was evidently seen in the form of large leaf, stem and basal galls formed on the susceptible plants. Conversely there was an increase in the level of genes contributing to auxin metabolism in the resistant inoculated plants, indicating that the resistant plant inhibits uncontrolled cell division and therefore fails to show symptoms of large gall formation. The increased expression of detoxifying enzymes and antioxidants seen in the resistant inoculated

plants indicate that there is production of reactive oxygen species and hypersensitive response (HR). This suggests that the resistant plant employs HR to overcome pathogen infection by ceasing the nutrient supply to the biotrophic pathogen. Thus the analysis of genes that were up-regulated in the resistant inoculated plants shows that a majority of these have functions associated with plant defense and others promote activation of plant defense indicating that the resistant plant employs these genes to overcome *U. maydis* infection.

In addition to the up-regulated genes we also studied the genes that were down regulated in maize plants on *U. maydis* infection. Although there are no reports indicating a correlation of down regulated genes with plant defense, we were interested to see if down regulation of genes had any effect on the pathogen. The most interesting findings were a significant down regulation of genes that had functions associated with photosynthesis and plant glycolysis in the resistant inoculated plants as compared to susceptible inoculated and control plants. The most likely explanation for the decrease in expression of these genes is to deplete the nutrient supply to the biotrophic pathogen.

In this study, a comprehensive list of transcripts regulated in resistant and susceptible inoculated and mock inoculated maize lines at 24 hpi following *U. maydis* inoculation has been identified. A distinct difference between the resistant and susceptible inoculated and mock inoculated comparisons was seen. Indicating that the resistant plant is capable of recognizing the presence of *U. maydis* and up-regulating genes involved in plant defense to overcome infection. This can be correlated to the resistant phenotype seen in the resistant maize lines that was characterized by chlorosis, anthocyanin and minute leaf gall formation. Contrary to this the susceptible maize lines did not show a significant increase in the

expression of genes associated with plant defense and were characterized by the presence of large leaf, stem, basal galls and death. This clearly shows that the resistant phenotype can be linked to the expression of genes involved in plant defense. The resistant plant recognizes the presence of pathogen and up-regulates plant defense genes to overcome infection, while it down regulates genes involved in photosynthesis and plant glycolysis to decrease the nutrient supply to the biotrophic pathogen. This clearly shows that the resistant plant recognizes the presence of pathogen and alters the expression of its genes to overcome pathogen infection. These findings provide new insights into the complex changes in plant gene expression occurring globally in response to *U. maydis* that has not been clearly explained in prior studies. In addition to this it gives us a better understanding of the plant defense mechanism in maize in response to *U. maydis* infection.

Understanding plant defense meachanisms will facilitate the identification of genes that are associated with resistance against *U. maydis.* The identification of plant defense genes from the microarray can be used to design gene-specific primers. The primers can be used to screen potential maize lines for resistance against *U. maydis*. This will be a fast and efficient method for screening for resistant plants, rather than growing the plants, inoculating them and scoring for resistance. Additionally, 10 days old plants can be grown in greenhouse, inoculated with *U. maydis* and phenotyped to identify resistant plants. Thus, the molecular method is not only faster it is also easier and a more reliable method to identify resistant maize lines.

The microarray results indicate that the resistant plant down-regulates genes associated with plant glycolysis and photosynthesis to decrease the nutrient supply to the

biotrophic pathogen. This information can potentially be used to modify the fertilizer application on infected maize plants. To elaborate, once infection has occurred the application of fertilizers should be either reduced in quantity or the number of applications should be reduced. This will regulate the vegetative growth of the plant and thereby reduce the nutrient supply to the plant which will help in overcoming pathogen infection.

In addition to understanding the plant defense mechanism we were also interested to identify new sources of resistance against *U. maydis* from the wild progenitor teosinte. This was done with the aim to improve the resistance of economically important crop, maize. Traditionally plant breeding has been used to improve resistance in most crops. This involves breeding resistance genes from a resistant cultivar into a susceptible cultivar. However in a number of crops the known genes for disease resistance are being used up as they are released in cultivars and then overcome by new races of a pathogen. In addition to this cultivated species often lack genes required by the plant breeder particularly genes for disease resistance (Knott 1971). Thus the use of closely related species to introgress resistance genes is being used as an alternative strategy to improve resistance of cultivar crops. However there have been no reports indicating the identification of resistance against *U. maydis* in the wild progenitor teosinte. Therefore to fill this gap of knowledge we have identified two new sources of resistance against *U. maydis* from the wild progenitor teosinte namely; *Zea mays luxurians* and *Zea mays diploperennis*. This is the first report of identifying potential new sources of resistance to *U. maydis* from the wild progenitor teosinte. These teosinte lines have the potential to be employed in breeding programs to improve the resistance of maize against *U. maydis*. In order to prove this we phenotyped the maize teosinte introgression lines (NILs) that was generated by crossing maize B73 and *Zea*

mays parviglumis (teositnte) to identify NILs that have high level of resistance against *U. maydis.* This is first report where we have identified two maize teosinte introgression lines (Z031E0068 and Z031E1068) that show high level of resistance against *U. maydis* when compared with the recurrent parent B73. This indicates that teosinte can be used in breeding program to improve the resistance of maize against *U. maydis.* Our results indicate that even after making crosses between maize and teosinte we are able to see the resistance against *U. maydis* in the progeny lines. Concluding, that teosinte can be used as breeding material for the genetic improvement of maize and can be extremely useful to provide durable resistance against *U. maydis.*

There was no prior information available regarding the resistance of the teosinte lines (*Zea mays luxurian, Zea mays diploperennis* and *Zea parviglumis*) used in for this work including the teosinte parental line (*Zea parviglumis*) used to generate the maize teosinte introgression lines (NILs). The parental teosinte line (*Zea parviglumis*) used to generate the maize teosinte introgression lines was completely susceptible to *U. maydis* infection. However, in the future we can improve the resistance of cultivated maize against *U. maydis* by using the resistant teosinte lines (*Zea mays luxurians* and *Zea mays diploperennis*) identified from this work to make controlled crosses.

There were two maize teosinte introgression lines (NILs) that show high level of resistance against *U. maydis* even though both the parents of this population namely; B73 and *Zea mays parviglumis* are susceptible. The plausible explanation for this is the combination of maize and teosinte genes in the maize teosinte introgression lines that is responsible for the resistant phenotype, since resistance against *U. maydis* is controlled by

multiple genes (Baumgarten *et al*., 2007). Additionally, there is also a possibility of gene interactions between the maize and teosinte gene that is most likely contributing to the resistant phenotype. Given that the resistance against *U. maydis* is controlled by multiple genes and plant defenses involves numerous pathways that are activated simuntaneously, the interactions between maize and teosinte genes present in the NILs is likely contributing to the resistant phenotype. The genotype analysis of the two resistant NILs indicated that the teosinte introgressed regions in Z031E0068 and Z031E1068 is on chromosome 9 and is 3.9 Mbp and 3.6 Mbp respectively. Thus we have identified two NILs with high level of resistance against *U. maydis* and narrowed down the teosinte introgressed region in them that is potentially contributing to the resistant phenotype. The introgressed regionin Z031E0068 was present on chromosome 9 and was 3.9 Mbp in size. The second NIL, Z031E1068 is a further selfed (BC4S4) line of Z031E0068 and has the teosinte introgression present on chromosome 1, 4, 5 and 9. Phenotype analyses of additional NILs with tesosinte introgressions in these overlapping regions were susceptible to *U. maydis* infection. However, there were no NILs with an introgression present only on chromosome 9. The presence of multiple introgressions in Z031E1068 indicates that there is a possibility that there may be multiple introgressions present in Z031E0068. These multiple introgressions were not identified using the SNPs becuase fewer SNPs (~800) were identified in Z031E0068 as compared to the 33,000 SNPs for Z031E1068. In future the use of additional SNPs in Z031E0068 can lead to the identification of multiple introgressions and also help to narrow the teosinte introgressed region to one chromosomal location. Once a single teosinte introgressed chromosomal region is identified, additional NILs containing these introgressed regions can be screened to confirm their involvement in contributing to the resistant

phenotype. Additionally, the teosinte introgressed region present in the resistant NIL can be sequenced to identify the teosinte genes that are present in this region and are most likely contributing to the resistant phenotype.

However, due to lack of sequence information from the teosinte lines we were unable to identify the teosinte genes that were present in the introgressed region. Therefore we used the sequenced B73 genome to identify the genes that are potentially present in this region and are most likely contributing to the resistant phenotype. Analysis of the B73 genome sequence indicated that there are a total of 162 genes present on chromosome 9 which is the region corresponding to the teosinte introgressed region in the two resistant NILs (Z031E0068 and Z031E1068). Out of these 162 genes, 90 genes are uncharacterized and 7 genes have functions associated with resistance. Five genes from a total of 7 genes were receptor like kinase and/or protein kinases, one gene had a leucine rich repeat (LRR) domain and one gene was a putative subtilase. These seven genes have functions associated with plant defense for example the receptor like kinase and/or protein kinases are associated with recognition of pathogen effectors, the LRR domains are characteristic of plant resistance genes and subtilase is a pathogenesis related protein that is responsible for activation of plant defense. These genes are likely contributing to the resistant phenotype. However there are more genes involved in the resistance reaction and these are most likely from the teosinte genome. But due to lack of sequence information from teosinte we can only speculate the potential genes that are most likely present in the introgressed region. A complete list of all the genes in the teosinte introgressed region of the resistant NILs will enable us to identify the teosinte genes that are contributing to the resistant phenotype. This is possible only by sequencing the teosinte introgressed region present in the resistant NILs.

Overall, the results from this research have identified new sources of resistance in teosinte against *U. maydis.* It also gave an insight on the processes by which maize plants defend themselves against *U. maydis* infection. These studies are crucial for the design of novel strategies to develop maize cultivars with high levels of resistance to *U. maydis*. Additionally, a broad understanding of the resistance genes present in maize and their relationships within the genome will provide a foundation for understanding and improving maize, as well as other cereal crops. Finally, this work could have major implications for characterization of a resistance mechanism that may be more durable and development of novel strategies to control *U. maydis* by dissecting plant-pathogen interactions.

The microarray analysis has presented a comprehensive list of differentially expressed genes in maize in response to *U. maydis* at 24hpi. In future it would be interesting to study these genes at different time points to obtain a better understanding of time dependent expression of these genes. This will give us a clear knowledge regarding the expression of plant defense genes at different time points in order to understand how much time the plant takes to recognize the presence of pathogen and the duration of defense mechanism which it employs to overcome infection. Apart from this the two newly identified teosinte lines (*Zea mays luxurians* and *Zea mays diploperennis*) that show high level of resistance against *U. maydis* can be used in breeding programs to improve the resistance of cultivated maize plants against *U. maydis.* This can be accomplished by generating near isogenic lines using either B73 or the resistant maize line (*Zea mays subsp. mays* NSL 30060, United States, Wisconsin) that we have identified in this study as the maize parent. The progeny lines generated by these crosses have the potential to inherit the resistance that is seen in teosinte parent (*Zea mays luxurians/ Zea mays diploperennis)*. Further there is a

possibility of improved resistance when *Zea mays subsp. mays* (NSL 30060, United States, Wisconsin) is used as the maize parent since both parents (*Zea mays luxurians/ Zea mays diploperennis, Zea mays subsp.mays*, NSL 30060, United States, Wisconsin) would be resistant. It would also be interesting to study the correlation of genes obtained from the microarray data in the two resistant NILs (Z031E0068 and Z031E1068). To elaborate, the sequence information of the up-regulated plant defense genes obtained from the microarray data can be used to design gene specific primers that can be used to PCR amplify these genes from the two resistant NILs. These genes can be cloned and sequenced to verify if they are the appropriate plant defense genes that were differentially expressed in the microarray data. In addition to this a RT-PCR using these genes in the two resistant NILs will confirm the expression of the plant defense genes. This will give us a list of genes that are expressed in the two NILs and are potentially contributing to the resistant phenotype. Lastly, a precise method of obtaining all the genes present in the teosinte introgressed region of the two resistant NILs (Z031E0068 and Z031E1068) that are contributing to the resistant phenotype is possible only by sequencing the teosinte introgressed region in these NILs.

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APPENDIX A

DIVERSITY AND EVOLUTION OF *RP1* RUST RESISTANCE GENES IN FOUR MAIZE LINES

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Abstract

The *Rp1* locus of maize is a complex resistance gene (R-gene) cluster that confers race-specific resistance to *Puccinia sorghi*, the causal agent of common leaf rust. *Rp1* NB-LRR disease resistance genes were isolated from two *Rp1* haplotypes (*HRp1-B* and *HRp1-M*) and two maize inbred lines (B73 and H95). Sixty-one *Rp1* genes were isolated from *Rp1-B*, *Rp1-M*, B73 and H95 with a PCR-based approach. The four maize lines carried from 12 to 19 *Rp1* genes. From 4 to 9 of the identified *Rp1* genes were transcribed in the four maize lines. The *Rp1* gene nucleotide diversity was higher in *HRp1-B* and *HRp1-M* than in B73 and H95. Phylogenic analysis of 69 *Rp1* genes revealed that the *Rp1* genes maintained in *HRp1-B*, *HRp1-M* and H95 are evolving independently of each other, while *Rp1* genes in B73 and *HRp1-D* appear more like each other than they do genes in the other lines. The results also revealed that the analyzed *Rp1* R-genes were under positive selection in *HRp1-M* and B73. Intragenic recombination was detected in *Rp1* genes maintained in the four maize lines. This demonstrates that a genetic process that has the potential to generate new resistance genes with new specificities is active at the *Rp1* locus in the four analyzed maize lines and that the new resistance genes may act against newly-arising pathogen races that become prevalent in the pathogen population.

Key Words: *Rp1*, resistance genes, NB-LRR, genetic diversity, R-gene evolution

Introduction

One vital trait that affects all plants grown in agricultural or natural environments is their ability to withstand disease. Plants have evolved several mechanisms of protection in response to pathogen infection. The plant defense response is one of these systems and involves an elaborate induction process following plant recognition of a pathogen avirulence gene product by a plant resistance gene product. The most abundant resistance genes are those that encode proteins with nucleotide binding site (NB) and leucine-rich repeat (LRR) domains (Bent 1996; Hulbert *et al*. 2001). To date, about three fourths of the plant disease resistance genes that have been cloned are from this class. Several conserved motifs are maintained in the NB domain and are responsible for nucleotide binding and initiating a signal transduction cascade to activate plant defenses (Tameling *et al*. 2002). The LRR region is typically involved in protein-protein interactions and pathogen recognition specificity (Leister and Katagiri 2000; Dangl *et al*. 2001; Jiang *et al*. 2007).

Despite these efforts, the pathogens ability to change or lose avirulence genes renders these genes unrecognizable by the corresponding plant resistance gene protein resulting in plant susceptibility. Therefore, the plant population must be able to produce new specificities in response to the changing pathogen in order to protect themselves and survive. More than 20 maize resistance genes were identified as *Rp* loci during the 1960's (Hooker and Russell 1962; Hagan and Hooker 1965; Saxena and Hooker 1968; Wilkinson and Hooker 1968). The majority of these genes mapped to a region on the short arm of chromosome 10. The *Rp1* complex consists of a highly variable cluster of fourteen (*Rp1-A* to *Rp1-F* and *Rp1-H* to Rp1-*N*) NB-LRR genes (Collins *et al*. 1999). Each of these genes represents a gene family that

can be distinguished by the *P. sorghi* isolates to which it confers resistance. Extensive genetic and molecular analysis of this locus has demonstrated that unequal intragenic (new gene) or intergenic (gene reassortment) crossing over events can create new resistance specificities (Richter *et al*. 1995; Smith and Hulbert 2004; Smith and Hulbert 2005; Smith *et al*. 2010). Therefore, *Rp1* is a classic example of a complex disease resistance locus. New resistance specificities have been selected in a few other systems. The best characterized system is the *L* locus of flax. Variant alleles were first characterized in flax in the early 1970s and were molecularly characterized more recently. Similar to the *Rp1* locus, it was found that the new resistances at the *L* locus were the result of recombination events between the two parental alleles involved in the cross-over event (Ellis *et al*. 1999; Ellis *et al*. 2000; Luck *et al*. 2000). The selection of variants with new resistant phenotypes from different systems demonstrates the significance of recombination in creating novel genes or haplotypes with new resistance specificities.

Comparative analysis of resistance gene family members has provided evidence that R-genes are subject to positive selection, particularly in the LRR region. The LRR region encodes solvent exposed residues that are predicted to interact with the corresponding *Avr* protein in the pathogen in a direct or indirect manner (Kobe and Deisenhofer 1995; Hu and Hulbert 1996). Mondragón-Palomino *et al*. (2002) demonstrated that selection has acted to diversify the LRR domain of several groups of *Arabidopsis* NB-LRR gene family members (Mondragón-Palomino *et al*. 2002). Additionally, comparative analysis of NB-LRR R-gene family members from tomato, lettuce, rice and flax has demonstrated that solvent-exposed residues of the LRRs are hypervariable (Mondragón-Palomino *et al*. 2002). This indicates that selective forces imposed by the pathogen incite allelic diversity (Hulbert *et al*. 2001). For

this reason, the selective advantage of carrying an R-gene and the pressure imposed on the R-gene to diversify depends on the frequency of the corresponding *Avr* gene in the pathogen population. Investigation of R-gene variation patterns has proven to be a powerful tool to estimate R-gene abundance and selection pressure.

Molecular analysis of the *Rp1* locus has contributed greatly to what is known about complex disease resistance loci and how they evolve. Analysis of R-genes in previously uncharacterized *Rp1* haplotypes will help identify resistance gene haplotypes that will be potentially important for the production of disease resistant varieties. The specific objectives of this study were to analyze the haplotypic diversity of NB-LRR R-genes in previously uncharacterized *Rp1* haplotypes and to postulate how these R-genes evolve. This study describes the diversity and evolution of *Rp1* R-genes from three *Rp1* haplotypes and two maize inbred lines. The findings provide insights into *Rp1* R-gene number, transcription and diversity, thus generating a data resource for future use of this class of genes for improved maize cultivar performance.

Materials and Methods

Plant material

Three *Rp1* haplotypes (*HRp1-B*, *HRp1-D* and *HRp1-M*) and two maize inbred lines (*B73*, *H95*) were selected for the analysis. The *Rp1* haplotypes were selected based on the predicted minimum number of *Rp1* genes maintained in each haplotype. Southern blot analysis indicated that *HRp1-B* and *HRp1-M* carry the fewest number of *Rp1* genes among the *Rp1* haplotypes that have not been subjected to sequence analysis (Collins *et al*. 1999).

Therefore, *HRp1-B* and *HRp1-M* were selected as additional *Rp1* haplotypes because fewer clones would need to be analyzed to sample all of the *Rp1* genes maintained in each haplotype. The *Rp1-D* haplotype is the best characterized *Rp1* haplotype and carries only nine *Rp1* genes (Sun *et al*. 2001). *Rp1* genes from the *Rp1-D* haplotype were used in this study for comparative analysis because all of the *Rp1* genes from this haplotype have been cloned and characterized and are a classic example of a resistance gene family. The three *Rp1* haplotypes are in the *H95* background, while many other previously characterized *Rp1* haplotypes are maintained in the *B73* background. Therefore, both *H95* and *B73* were selected for this work to analyze the *Rp1* genes in the two maize inbred lines. All *Rp1* haplotypes are near-isogenic. Seeds from the five maize lines were collected and planted in pots in the greenhouse to collect tissue for DNA and RNA extractions.

Genomic DNA and total RNA was isolated from fully expanded uninoculated second leaf sections collected from the four maize lines (*HRp1-B*, *HRp1-M, B73* and *H95*) using CTAB extraction methods with modifications (Murray and Thompson 1980) and GIBCOBRL (Rockville, MD) Trizol reagent as described by the manufacturer, respectively. Four seedlings were collected for each maize line and combined for DNA and RNA extractions. The quality of the DNA and RNA samples were assessed on a .5% agarose gel and the quantity was determined with the Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies).

PCR amplification and cloning of *Rp1* genes

A PCR based approach was used to isolate the C-terminal half of LRR region (1.0 kb) of *Rp1* genes from the four maize lines. *Rp1* genes are partially conserved in this region and

highly duplicated allowing for the design of highly conserved primers that will amplify the majority if not all of the *Rp1* genes in a haplotype (Sun *et al*. 2001; Smith *et al*. 2004; Smith *et al*. 2010). This method facilitates high fidelity amplification of large target lengths (.1-48 kb) and has proven to be very efficient when amplifying the 4 kb coding region of many *Rp1* genes (Sun *et al*. 2001; Smith *et al*. 2004; Smith *et al*. 2010). PCR amplification of genomic DNA templates was performed with Enhanced DNA Polymerase (Stratagene, La Jolla, CA) with approximately one minute of extension time for every kb of fragment size. All other parameters were performed according to the manufacturer's suggestions. The DNA's were amplified with a conserved primer pair (Forward P19-TTGATAGGTTGGTTGTAAGTG; Reverse 4890R-CCTGAACTCTGGAGCTTCAAC) designed from the LRR and 3´ UTR region. This region has been used in the design of PCR-based cloning to characterize R-genes from dicot and monocot species (Meyers *et al*. 2003). The resulting 1.0 kb PCR products were isolated from a 1.5% agarose gel, purified with an Invitrogen Quick gel extraction kit (Carlsbad, CA) and cloned into the Invitrogen (Carlsbad, CA) TOPO TA cloning vector using the methods described by the manufacturer. One library for each *Rp1* haplotype (*Rp1-B* and *-M*) and maize inbred line (H95 and B73) was generated from each cloning experiment. Ninety-six to one-hundred-and-ninety-two clones were sequenced with T3 and T7 primers per gel purification product using the Big-Dye Terminator v3.1 cycle sequencing kit (Applied Biosystems), following the manufacturer's protocol. All sequencing was performed at the UGA sequencing facility.

Analysis of *Rp1* transcripts

RT-PCR was performed on 500ng of total RNA isolated from the two *Rp1* (*Rp1-B* and *Rp1-M*) haplotypes and two maize inbred lines (B73 and H95) to indentify the transcribed *Rp1* genes. *Rp1* transcripts have been characterized previously from the *Rp1-D* haplotype. *Rp1* cDNA sequences were amplified using a StrataScript First-Strand Synthesis System as described by the manufacturer (Stratagene, LaJolla, CA). Following first strand synthesis with an oligo dT primer, second strand synthesis was performed using a primer pair (4065F-TGCCATGAGCAGAGGATAAGAT/4890R-CCTGAACTCTGGAGCTTCAAC) designed from a conserved region among *Rp1* genes that flanks a highly polymorphic region at the 3´ end of *Rp1* genes to differentiate between *Rp1* sequences. The primer pair also flanks a 3´ intron for detection of genomic DNA contamination. The resulting 1.2 kb cDNA product was gel purified and cloned into the Invitrogen (Carlsbad, CA) TOPO TA cloning vector. Ninety-six to one-hundred-and-ninety-two cDNA clones were sequenced from each *Rp1* haplotype and maize inbred line with T3 and T7 primers (Table A.1). The PCR amplified genomic and cDNA clones overlap in the C-terminal half of the LRR region. Therefore, the cDNA sequences with homology to *Rp1* genes were aligned to the *Rp1* genomic sequences isolated from the *Rp1* haplotypes and maize inbred lines to identify the transcribed *Rp1* genes.

Sequence diversity and recombination analysis

Rp1 genomic and cDNA sequences isolated from the two *Rp1* haplotypes and two maize inbred lines were used to run a BLASTN search against the NCBI non-redundant database to verify putative homologies to known resistance genes. *S*equences from genomic

and cDNA clones were aligned separately to identify the overlapping region and to assemble the LRR region of each clone. The LRR region of each genomic and cDNA clone was aligned to identify redundant clones, estimate the number of *Rp1* genes maintained in the four maize lines and determine how many *Rp1* gene are transcribed in each maize line. Due to the polymorphic nature of the LRR region of *Rp1* genes, this region has been particularly useful when distinguishing between *Rp1* genes and estimating gene number (Smith *et al*. 2010).

The accuracy and assembly of *Rp1* sequences were verified with PHRED and PHRAP bioinformatics tools, respectively (Ewing and Green 1998) as well as BioEdit (Hall 1999). The minimum value for acceptable sequences was set at 20 (q>20) for a PHRED quality score, or an accuracy of 99.99% (Ewing and Green 1998). The accuracy of single nucleotide polymorphisms was further verified by scrutinizing chromatograms. Additionally, two identical clones from different PCR reactions were sequenced and aligned to identify nucleotide polymorphisms introduced by PCR. Sequences were manually edited when errors were found to be introduced. The ends of sequences were trimmed when sequences did not meet the minimum value for a PHRED quality score (q>20). All sequencing was performed in the Genomics and Bioinformatics Sequencing Facility at the University of Georgia.

DnaSP (DNA Sequence Polymorphism) software package version 5.10.01 (Librado and Rozas 2009) was used to estimate the sequence diversity of *Rp1* genes by calculating the average pairwise difference between sequences, π (Tajima 1983) and the number of segregating sites in a sample, θw (Watterson 1975). The latter parameter has expected values of $4Ne\mu$ for an autosomal gene of a diploid organism, where *Ne* and μ are the effective

population size and the mutation rate per nucleotide site per generation, respectively. Total sequences and silent sites were considered separately for nucleotide diversity estimates (Librado and Rozas 2009). The recombination parameter (*R*) per gene and between adjacent sites was calculated based on the average number of nucleotide differences between pairs of sequences (Hudson *et al*. 1987). The recombination parameter (*R*) has expected values of $4Nr$ for an autosomal gene of a diploid organism, where *N* is the population size and *r* is the recombination rate per sequence per gene.

Tajima's *D* analysis was performed using DnaSP and PAML (Yang 1997; Yang *et al*. 2000) to test for deviations from the neutral equilibrium model of evolution. Tajima's *D* is based on the discrepancy between the mean pairwise differences (π) and Watterson's estimator (θ_w) (Tajima 1989), and was calculated for *Rp1* genes in each maize line at all sites and at silent sites separately.

A phylogenetic tree was constructed with *Rp1* genomic sequences by MEGA 5 (Nei 1987; Saitou Nei 1987; Tamura *et al*. 2007; Tamura *et al*. 2011) using the Neighbor-Joining (NJ) method with distances represented as the number of nucleotide differences. One thousand bootstrap replicates were used to assess the confidence of the phylogeny. Neighbor-joining tree construction of *Rp1* genes from each maize line facilitated the analysis of sequence diversity and the distribution of *Rp1* genes into haplotypes.

Results

Estimate of *Rp1* genes and transcribed members in the four maize lines

To estimate the number of *Rp1* genes maintained in the analyzed maize lines and to identify the transcribed *Rp1* genes, two 3´ end primer pairs (P19/4890R; 4065F/4890R) designed from conserved regions within the C-terminal end of the LRR domain of characterized *Rp1* genes was used to amplify genomic and cDNA template from *HRp1-B*, *HRp1-M*, B73 and H95. The use of a conserved primer pair allows for the amplification of the majority, if not all, of the *Rp1* genes in a haplotype. Additionally, the C-terminal end of the LRR region was selected for this work because PCR primers could be designed to generate an amplification product that would cover the most divergent region of *Rp1* genes and where recombination most frequently occurs (Lawrence *et al*. 1997; Ellis *et al*. 2000). This would alleviate the need to sequence complete *Rp1* genes that are ~4kb in size from maize lines that likely carry a large number of *Rp1* genes. A PCR amplified fragment ~1.0 kb in size corresponding to the divergent C-terminal end of the LRR region was isolated and cloned as putative *Rp1* resistance genes. When *Rp1* clones were sequenced from each haplotype, multiple clones corresponded to individual *Rp1* genes, suggesting that the majority of the *Rp1* genes in these haplotypes had been sampled efficiently. It is possible that there are more than the identified *Rp1* genes in each haplotype, since genes that are identical through the sequenced ~1.0 kb region would not be differentiated.

A total of 544 genomic and cDNA clones were sequenced for the four maize lines (Table A.1). From these clones, 531 were observed to be homologous to the LRR sequences of *Rp1* and other NB-LRR encoding genes previously isolated from maize and other plant

species. A 79bp intron was present between the conserved primer sites and was used to detect genomic contamination in the cDNA sequences. Alignment of the genomic and cDNA sequences demonstrated that the 3' intron had been removed from the cDNA sequences indicating the absence of genomic contamination. The *Rp1* sequences were designated *Rp1-B-p*, *Rp1-M-p*, *Rp1-B73-p* or *Rp1-H95-p*. The *Rp1* designation indicates the locus and is followed by the name of the maize line the *Rp1* genes were isolated from. The p designation corresponds to the assigned paralog number (Table A.1).

One hundred and ninety two of the 531 PCR amplified clones were genomic clones isolated from the *Rp1-B* haplotype. Alignment of the *Rp1-B* genomic sequences and removal of redundant sequences identified sixteen different sequences representing unique *Rp1* genes and were designated as such (Table A. 1). The sixteen *Rp1* genes were aligned with the one hundred and ninety two *Rp1-B* cDNA sequences to identify the transcribed *Rp1-B* genes. The *Rp1-B* cDNA sequences corresponded to eight of the sixteen *Rp1-B* sequences indicating, *HRp1-B* carries at least sixteen unique *Rp1* genes, eight of which are transcribed. The eight LRR encoding *Rp1-B* genes had uninterrupted ORFs after the 3´ intron was removed and were identical to their corresponding cDNA sequences. The remaining eight *Rp1-B* genes did not correspond to any of the cDNAs and harbored stop codons or frame shift mutations. These genes were therefore considered untranscribed *Rp1* genes and are likely pseudogenes.

Ninety-six PCR amplified genomic and cDNA clones were isolated from *HRp1-M*. Based on sequence alignment, nineteen different *Rp1* genes were represented among the ninety-six clones (Table A.1). The *HRp1-M* cDNA sequences corresponded to twelve of the genomic sequences (Table A.1). Therefore, *HRp1-M* carries at least nineteen unique *Rp1*

genes, twelve of which are transcribed. The transcribed genes contained uninterrupted ORFs and were identical to their corresponding cDNA sequences with the exception of the 3´ intron. Seven of the *Rp1-M* genes contained stop codons and/or frame shift mutations.

Alignment of ninety-six genomic and cDNA sequences isolated from B73 identified fourteen different sequences (Table A.1). The B73 cDNA sequences were identical to four B73 genomic sequences indicating B73 carries at least fourteen different *Rp1* genes and four of these genes are transcribed members. One-hundred-and-sixty genomic and cDNA clones were analyzed for the H95 maize line. The clones represented twelve different *Rp1* genes (Table A.1). Eight of these genes are transcribed. All of the transcribed *Rp1* genes isolated from B73 (4 genes) and H95 (8 genes) contained uninterrupted ORFs, while the untranscribed *Rp1* genes harbored stop codons or frame shift mutations.

Rp1 nucleotide and haplotype diversity

The LRR region of *Rp1* genes was analyzed in *HRp1-B, HRp1-M, B73* and *H95* using DnaSP (Librado and Rozas 2009) to determine the genetic diversity of these genes at a complex disease resistance locus. The length of aligned sequence for *Rp1* genes from the four maize lines varied between 696 bp and 754 bp and contains only coding sites. This variability was due to manual trimming of the ends of sequences that did not meet the minimum value for a PHRED quality score (q>20). *HRp1-B, HRp1-M, B73* and *H95* maize lines exhibited 342, 351, 52 and 371 SNPs, respectively (Table A.2). Although numerous indels and SNPs were detected, thirty-nine (52%) of the total number of different *Rp1* genes (61) identified in the four maize lines appeared fully functional and were transcribed. For the remaining twenty-two *Rp1* genes (48%), frame shifts or SNPs yielding premature stop

codons suggested nonfunctional alleles, at levels of 50% (8 of 16 *Rp1* genes are nonfunctional), 37% (7 of 19 *Rp1* genes are nonfunctional), 71% (10 of 14 *Rp1* genes are nonfunctional) and 33% (4 of 12 *Rp1* gene are nonfunctional) in *HRp1-B, HRp1-M, B73* and *H95*, respectively.

The average nucleotide diversity (π) for *Rp1* genes in *HRp1-B, HRp1-M, B73* and H95 was 2.28%, 3.31%, .51% and 1.35%, respectively (Table A.2). The number of segregating sites detected in *HRp1-B, HRp1-M,* B73 and H95 was 342, 351, 52, and 371, respectively. Higher nucleotide diversity was observed in the LRR of *Rp1* genes maintained in the *Rp1* haplotypes (*Rp1-B* and *-M*) in comparison to *Rp1* genes isolated from *B73* and *H95*. Similar estimates of diversity were also observed for *HRp1-B* (2.03%), *HRp1-M (*2.00%) and B73 (.48%) with the θ_w parameter, while the *Rp1* genes in H95 (2.09%) demonstrated a higher nucleotide diversity with this measurement (Table A.2). Watterson's estimator (θ_w) is a method used for estimating population mutation rate (genetic diversity) but also takes into account the effective population size and the mutation rate per-generation in the population of interest, whereas the π estimator is simply the sum of the pairwise differences divided by the number of pairs.

Neighbor-joining tree construction for *Rp1* genes isolated from *Rp1-B, Rp1-M, B73* and *H95* facilitated the analysis of haplotypic diversity relative to the LRR region. This analysis showed the distribution of *Rp1* genes from each maize line into clusters based on sequence differences. Clones were defined as belonging to a cluster (closely related family of genes) based on the nucleotide sequence identity of the dataset, when aligned sequences demonstrated at least 90% nucleotide identity. *Rp1* genes present in different clusters were

less than 70% identical. This was not surprising because R-gene homology represents true relatedness. Different clusters represent different *Rp1* haplotypes (*H*). The *Rp1* gene haplotype relationships were well-supported within the clusters indicated by the high bootstrap support for all of the clades (Figure A.1 thru A.4). There were no distinct classes formed in any of the neighbor-joining trees based on transcribed *Rp1* genes.

The *Rp1* maize lines carried a relatively large number of *Rp1* genes ranging from 12 (*H95*) to 19 (*HRp1-M*) unique genes and were arranged into a large number of haplotypes (Figures A.1 thru A.4). Indels were identified in *Rp1* genes isolated from each haplotype. Indel sizes in the untranscribed *Rp1* genes were variable, with single nucleotide indels being the most frequent size class.

The sixteen *Rp1* genes identified in *HRp1-B* were distributed into ten distinct clusters based on nucleotide sequence identity and represented different *Rp1* haplotypes (Table A.1). The most distant *Rp1* genes were *B-p19** and *B-p24* (Figure A.1 and Table A.1). These two genes were separated by 365 polymorphic sites in a 707 bp region and are 48.1% identical. Conversely, *Rp1-B-p66* and *Rp1-B-p65* differed by a single nucleotide substitution and share 99.8% identity.

Similarly, the nineteen *Rp1-M* genes formed eleven different clusters and represented different *Rp1* haplotypes (Figure A.2 and Table A.1). *Rp1-M-p1** and *Rp1-M-p14** were the most distant with 351 nucleotide differences (49.1% identity) in a 690 bp region. Paralogs *Rp1-M-p3** and *Rp1-M-p10** were the most similar, separated by only one nucleotide change (99.7% identity).

The fourteen *Rp1* genes identified in B73 were distributed into ten *Rp1* haplotypes (Figure A.3 and Table A.1). The most distant *Rp1* paralogs were *B73-p13* and *B73-p45**. These two paralogs were separated by 52 polymorphic sites in a 615bp region and are 92% identical. The *Rp1-B73-p16* and *Rp1-B73-p6** paralogs differed by two nucleotide substitutions and share 99.6% identity.

Analysis of H95 *Rp1* sequences identified twelve *Rp1* genes that formed eight *Rp1* haplotypes (Figure A.4 and Table A.1). *Rp1-H95-p1* and *Rp1-H95-p50** were the most distant *Rp1* genes differing by 371 single nucleotide substitutions in a 708bp region thus are 47% identical. However, *Rp1-H95-p29** and *Rp1-H95-P63* were the most similar separate by only two single nucleotide substitutions and share 99.6% identity.

A composite neighbor-joining tree was constructed with the 70 *Rp1* genes identified in the *HRp1-B, HRp1-M, B73, H95* and *HRp1-D* (Figure A.5). The *Rp1* genes were separated into four distinct clades with 33, 8, 14 and 14 genes clustering together in clade I, II, III, and IV, respectively. The majority of the *Rp1* genes from *HRp1-B, HRp1-M,* and H95 formed clade I. Thirteen of the sixteen *Rp1-B* genes clustered in clade I and eleven of the twelve *Rp1* genes from H95 were identified in this clade. Eight of the nineteen *Rp1-M* genes were also present in clade I, while there were no *Rp1* genes from B73 identified in clade I. Similarly, clade III contained *Rp1* genes from *Rp1-B, -M* and *H95*. The remaining 3, 10 and 1 *Rp1* genes from *HRp1-B, HRp1-M* and H95 respectively, were clustered in clade III, while B73 *Rp*1 genes were absent from this clade. There were two monophyletic clades (II and IV) formed containing *Rp1* genes from the same haplotype. Clade II contains only *Rp1-D* genes from *HRp1-D*. *HRp1-D* is the best characterized *Rp1* haplotype and was used in this analysis

to determine the evolutionary relationship of known *Rp1* genes to uncharacterized *Rp1* genes. Additionally, all 14 of the B73 *Rp1* genes clustered in clade IV. Identical *Rp1* genes were not identified in *HRp1-B*, *HRp1-M*, B73 and H95.

The most distant *Rp1* genes among the five haplotypes were *H95-p61* and *B73-p13*. These two genes were separated by 310 polymorphic sites within a 642bp region and share 51.7% identity. Conversely, *B-p36** and *M-p25** *Rp1* genes in clade III differed by four nucleotide substitutions and are 99.4% identical.

The nucleotide diversity data were also supported by phylogenetic analysis of the *Rp1* genes characterized in the four maize lines (Figure A.5). As indicated, the *Rp1* genes isolated from B73 all clustered on clade IV. These genes were also the least divergent of the characterized *Rp1* genes based on sequence analysis. This clade was further divided into two smaller clades with short branch lengths. Similarly, *Rp1* genes from H95 also demonstrated low nucleotide diversity and clustered on a single clade with *Rp1* genes from *HRp1-B* and *HRp1-M* (Clade I) with the exception of paralog *H95-p50**. Conversely, *Rp1* genes from *HRp1-B* and *HRp1-M*, the more divergent haplotypes, did not cluster into monophyletic clades.

Detection of recombination and positive selection.

The frequency of recombination in the LRR region of the *Rp1* genes from the four maize lines was examined by using the recombination parameter (*Rm*) (Hudson *et al*. 1987) from DNAsp (Table A.3). The minimum number of recombination events between adjacent polymorphic sites for *HRp1-B*, *HRp1-M*, B73 and H95 were 19, 22, 3 and 9, respectively.

The recombination frequency (between adjacent sites and per gene) was relatively low for all *Rp1* haplotypes analyzed with the highest values observed for *HRp1-M* (0.0023 and 1.5) subsequently decreasing for *HRp1-B* (0.0012 and 0.001), B73 (0 and 0.001), and *H95* (0 and 0.001).

Patterns of nucleotide substitution in the LRR region of R-genes can be informative in assessing the type of selection pressure acting on the evolution of gene family members (Sun *et al*. 2001). Nucleotide diversity was detected at 0.43984 in the LRR region of the 69 *Rp1* genes. Nonsynonymous (K_a) and synonymous (K_s) amino acid substitution rates for the 69 *Rp1* genes were 1.66518 and 0.65045, respectively. Therefore, the nonsynonymous to synonymous amino acid substitution ratio (K_a:K_s) for the 69 *Rp1* genes is >1 (2.56003) indicating that the LRR region of *Rp1* genes maintained in the four maize lines are under positive selection. Tajima's *D* statistics was also used to detect neutral selection. When the *Rp1* genes from each maize line were tested separately with Tajima's *D* tests, negative values were observed for *HRp1-B* and H95 with no selection detected with significant *P* values, indicating a relative excess of low frequency alleles compared with expectations under a stationary neutral model (Table A.3). Conversely, positives values were detected for *HRp1-M* and B73 with Tajima's *D*, with significant *P* values detected.

Discussion

Amplification of *Rp1* genes from four maize lines

Extensive studies on plant disease resistance genes have demonstrated that resistance genes frequently occur in tightly linked clusters (Pryor 1987; Michelmore and Meyers 1998).

Multiple *Rp* (Resistance to *Puccinia sorghi)* genes have been shown to confer resistance to *P. sorghi* in maize. This locus was designated the *Rp1* complex because fourteen genetically distinct loci mapped to this locus on the short arm of chromosome 10 in maize (Hulbert 1997). Complex disease resistance clusters have also been identified in Arabidopsis (Meyers *et al*. 2003), rice (Song *et al*. 1997), barley (Wei *et al*. 1999) and many other species. Many of the specificities within these genetically well-defined resistance loci have been targeted for molecular cloning and analysis utilizing a PCR-based approach and/or genomic library screening methods. In this study, a PCR-based approach was used to analyze two previously uncharacterized *Rp1* maize lines and two maize inbred lines by estimating *Rp1* gene number and diversity, identification of transcribed *Rp1* genes and detection of recombination and selection acting on these genes in the four maize lines. The PCR based approach amplified the C-terminal end of LRR region of *Rp1* genes in the four maize lines. This is the most divergent region of *Rp1* genes and has been used to distinguish between *Rp1* genes and to estimate R-gene number (Richter *et al.* 1995; Smith *et al.* 2004; Smith *et al.* 2005; Smith *et al.* 2010) without sequencing the full-length gene as most *Rp1* genes are ~4kb in size. This approach did not distinguish between identical genes or amplify truncated genes in this study. As a result, these types of *Rp1* genes were not sampled. Hence, to study specific haplotypes that carry an abundance of large R-genes, a PCR-based approach has proven to be most appropriate, where single molecules represent pertinent regions of each haplotype.

From this work, 544 PCR amplified sequences corresponding to sixty-one R-genes isolated from *HRp1-B*, *HRp1-M*, *H95* and *B73* were homologous to the LRR sequences of *Rp1* and other NB-LRR encoding genes. The number of *Rp1* genes maintained in each maize line is relatively large ranging from at least 12 to 19 genes. Based on Southern blot analysis

and characterization of *Rp1* genes in various *Rp1* maize lines, most *Rp1* lines carry from 15 to 25 *Rp1* genes and are considered large haplotypes (Ayliffe *et al*. 2000; Sun *et al*. 2001; Ayliffe *et al*. 2004; Smith *et al*. 2010). Additionally, genomic sequence analysis of maize BAC clones from a B73 maize inbred line identified 15 *Rp1* genes (Ramakrishna *et al*. 2002), whereas this study identified 14 *Rp1* genes in a different B73 maize inbred line using a PCR-based approach. Analysis of *Rp1* genes in the *HRp1-A* and *HRp1-K*, using a similar PCR-based approach, identified more than 50 *Rp1* genes in these haplotypes, while *HRp1-A188* carries a single *Rp1* gene (Smith and Hulbert 2004). This suggests that similar to most maize lines that carry *Rp1* genes, *HRp1-B*, *HRp1-M*, *H95* and *B73* also maintain a relatively large number of *Rp1* genes. Extensive studies in maize, flax and tomato have suggested that the large number of resistance genes maintained in tandem at complex disease resistance loci play a central role in the diversity and evolution of new specificities (Parniske *et al*. 1997; Ellis *et al*. 1999; Luck *et al*. 2000; Chin *et al*. 2001).

Rp1 nucleotide and haplotype diversity in four maize lines

Sixty-one *Rp1* genes were sampled from four maize lines. Various diversity patterns were detected within the LRR region of the *Rp1* genes maintained in the four maize lines. The LRR region of *Rp1* genes from B73 and H95 harbored the lowest diversity, while *Rp1* genes from *HRp1-B* and *HRp1-M* demonstrated the highest level of diversity between the four maize lines. Many factors can affect genetic diversity including, population size relevant for all genes and pathogen populations that are specific to each R-gene. Therefore, the higher diversity observed in *HRp1-B* and *HRp1-M* may partly reflect increased diversity in the LRR region as a mechanism of adaptive plasticity for disease resistance and responses to other

environmental variables (Clay and Kover 1996). For example, a more diverse set of *P. sorghi* isolates recognized by the *Rp1* genes from *HRp1-B* and *HRp1-M* may have been prevalent in the pathogen population contributing to diversity in the *Rp1* haplotypes.

Identification of transcribed *Rp1* genes

A total of sixty-one *Rp1* genes were identified in the four maize lines, thirty-two of these genes are transcribed. Presumably, the maize lines with large numbers of *Rp1* genes have more genes that are transcribed, but this has not been demonstrated. *HRp1-B* and *HRp1-M* carry the most *Rp1* genes, whereas B73 and H95 carry the fewest. Interestingly, the majority of the *Rp1* genes in each of the maize lines are transcribed with the exception of B73. All untranscribed *Rp1* genes harbored stop codons or frame shift mutations. This is a novel observation that has been observed at the *Rp1* locus with a few other haplotypes including *HRp1-E, HRp1-I* and *HRp1-K* (Smith *et al*. 2004). Based on the co-evolution of plant R-genes and pathogen *Avr* genes, selection pressure is imposed on the pathogen to evolve new genotypes (*Avr* gene) that can avoid detection by the corresponding plant R-gene protein. This implies that the nonfunctional *Rp1* alleles were potentially generated as a result of the complex plant-pathogen co-evolutionary dynamics and selection. It is possible that there is selection against nonfunctional transcripts at disease resistance loci and that the nonfunctional *Rp1* genes became ineffective due to mutation or defeat by a new race of the pathogen.

It has also been shown that the nonfunctional resistance genes participate in recombination creating new resistance genes (Hulbert 1997; Hulbert *et al*. 2001; Smith *et al*. 2004). For example, recombinants selected for complete or partial loss of *Rp1-D* resistance

resulted from unequal crossing over that occurred mostly within coding regions. The *Rp1-D* gene was altered or lost in all recombinants. The majority of recombination events involved the same untranscribed paralog with the functional *Rp1-D* gene. One recombinant with a complete LRR from *Rp1-D*, but the amino-terminal portion from an untranscribed paralog, conferred the *Rp1-D* specificity but with a reduced level of resistance. This indicates the potential usefulness of the nonfunctional genes in the creation of new resistance specificities at the *Rp1* locus.

Phenotypic analysis of *HRp1-B* and *HRp1-M* indicates that these *Rp1* haplotypes confer race-specific resistance to a different set of *Puccinia sorghi* rust isolates (Richter *et al*. 1995). Therefore, *Rp1* haplotypes are designated by the genes they carry with detectable phenotypes. However, B73 and H95 are susceptible to all known *P. sorghi* isolates but still carry transcribed *rp1* paralogs. This suggests that although *Rp1* lines typically carry a large number of *Rp1* genes that are often transcribed, most of these genes do not confer a resistance phenotype to any known rust isolate. This is also demonstrated in the *Rp1-D* haplotype. *HRp1-D* is the best characterized *Rp1* haplotype and is considered one of the most meiotically stable of the fourteen different *Rp1* haplotypes (Collins *et al*. 1999). This haplotype contains nine paralogs, seven of which are transcribed, including a truncated gene. However, only one paralog (*Rp1-D*) is phenotypically detectable and is located on the most distal end of the array. It is plausible that the transcribed paralogs in *Rp1* haplotypes are involved in other aspects of resistance that are not phenotypically detectable or these genes once conferred resistance to a *P. sorghi* isolate that no longer exists in the pathogen population but are still transcribed (Jullien and Berger 2009).

Evidence of positive selection and recombination

The LRR region of *Rp1* genes from the maize lines were determined to be under positive selection. Sequences of the *Rp1* genes corresponded to the C-terminal end of the LRR region and ranged from 696 to 754 bp in size. Tajima's *D* tests were also applied separately to the LRR region of *Rp1* genes from each maize line and indicated that positive selection had occurred in the C-terminal half of the *LRR* region of *Rp1* genes from *HRp1-M* and B73. This suggests that selection pressure favors diversifying selection in the C-terminal half of the LRR region of *Rp1* genes from *HRp1-M* and B73, which is consistent with the predicted function of the LRR domain of R-genes. There were no sites detected as under positive selection for *HRp1-B* and H95 *Rp1* genes. A relatively, high level of nucleotide diversity was observed for *Rp1-B* genes. This haplotype also confers race-specific resistance to different *P. sorghi* isolates, yet positive selection was not detected in the C-terminal end of LRR region, suggesting that regions other than the LRR of *Rp1-B* genes may be under positive selection and contribute to the resistance specificity.

The LRR domain is well documented as a functional structure involved in protein-protein interactions binding pathogen derived *avr* factors directly or indirectly (Kobe and Deisenhofer 1995; Ellis *et al*. 1999; Ellis *et al*. 2000). Over the past ten years, numerous studies have demonstrated that the LRR of R-genes are subject to positive selection and is where diversifying selection plays a role in the generation of new resistance specificities (Hu and Hulbert 1996; Parniske *et al*. 1997; Ellis *et al*. 1999; Hulbert and Drake 2000). There are also examples of regions other than the LRR that contribute to resistance specificity. One of the best-characterized examples is the *L* locus in flax. Analysis of this locus indicated that

the TIR (Toll Interleukin-1 Receptor) domain contributes to resistance specificity and may be under positive selection (Luck *et al*. 2000). To date, R-genes with a TIR domain have not yet been identified in grasses. Further evidence suggests that selection pressure acts differently on different regions of the LRR domain. For example, Jiang *et al.* (2007) found that 12 NB-LRR resistance gene loci demonstrated a significant Ka:Ks value at the C-terminal region of the LRR. In contrast, the N-terminal region of the LRR flanking the ß-strand/ß-turn motif (××L×L××) showed a conservative evolution.

Variability in the frequency of recombination events between the *Rp1* genes in the different maize lines was detected. This indicates that there are different histories of sequence exchange between *Rp1* genes in the different maize lines. Evidence has accumulated suggesting that unequal recombination is a major mechanism in diversifying R-gene sequences, especially at complex disease resistance gene loci (Sudupak *et al*. 1993; Parniske *et al*. 1997; Dixon *et al*. 1998; Hulbert *et al.* 2001; Ramakrishna *et al*. 2002; Nagy and Bennetzen 2008; Baurens *et al*. 2010). Recombination has been shown to contribute significantly in the creation of genetic diversity at the *Rp1* rust resistance locus (Hulbert *et al.* 1997; Smith *et al*. 2010). For example, phenotypic and genetic analyses of several recombinant *Rp1* haplotypes demonstrated the creation of novel recombinant *Rp1* genes and race specificities (Smith and Hulbert 2005; Smith *et al*. 2010). The generation of recombinant resistance genes that presumably create novel specificities has also been observed in flax, lettuce and tomato suggesting recombination plays a pivotal role in the evolution of new specificities (Parniske *et al*. 1997; Ellis *et al*. 1999; Luck *et al*. 2000; Chin 2001). The varibility of *Rp1* genes in different maize lines is likely due to two major contributing factors. First, there is likely a difference in selection frequency for recombined

alleles. This difference is perhaps due to different levels of pathogen stress imposed on the different haplotypes. Second, there are different intrinsic levels of genetic instability of *Rp1* genes in the different *Rp1* maize lines (Bennetzen *et al*. 1988). This genetic instability of *Rp1* genes may be due to where the *Rp1* gene with the phenotype occurs in the array, or how big the array is.

Phylogenetic analysis of *Rp1* genes

A neighbor joining tree was constructed for the *Rp1* genes isolated from each maize line to analyze the relationship between *Rp1* genes. From eight to eleven different *Rp1* haplotypes were detected for the four maize lines. However, only two distinct clades were observed for each maize line. The B73 and H95 clades demonstrated short branches within and between the different clades, while *Rp1-B* and *Rp1-M* branches were long in both instances. This suggests that the LRR region of *Rp1* genes is more diverse in *HRp1-B* and *HRp1-M* in comparison to B73 and H95. It has been shown in numerous studies that the LRR region is typically the most diverse domain in an NB-LRR resistance gene due to its involvement in pathogen recognition and specificity (Jiang *et al*. 2006; Sela *et al*. 2009). *HRp1-B* and *HRp1-M* confer resistance to different *P. sorghi* isolates. Therefore, these two haplotypes carry the appropriate *Rp1* gene(s) that recognizes the corresponding *Avr* protein in the pathogen population (Hammond-Kosack and Jones 1997). Conversely, B73 and H95 are maize inbred lines that are susceptible to all known *P. sorghi* isolates lacking the appropriate *Rp1* gene. It is possible that the most diverse *Rp1* genes observed in *HRp1-B* and *HRp1-M* arose most recently from recombination events in haplotypes with divergent *Rp1* arrays. However, the least diverse *Rp1* genes identified in B73 and H95 may be due to cyclical

amplification and deletion events that would homogenize haplotypes, especially in inbred populations, small populations or populations where a very successful haplotype became more prevalent.

A composite neighbor joining tree was generated using the 70 *Rp1* genes from the five maize lines to evaluate the evolutionary relationship between the genes from the different lines. This analysis included *Rp1* genes from the *Rp1-D* haplotype. Previous work at the *Rp1* locus demonstrated that *Rp1* genes characterized in different haplotypes vary in their evolutionary relationships and that their evolutionary patterns can be used to predict how the *Rp1* genes are evolving in individual haplotypes. For this study, *Rp1* genes from the five maize lines formed four distinct clades. *Rp1* genes from *HRp1-B*, *HRp1-M* and H95 shared two clades and were distributed between clades I and III. This suggests that *Rp1* genes maintained in *HRp1-B*, *HRp1-M* and H95 are more similar to each other than to *Rp1* genes within each individual maize line. Therefore, the *Rp1* genes maintained in *HRp1-B*, *HRp1-M* and H95 are evolving independently of each other.

Rp1 genes isolated from *HRp1-D* and B73 each formed a monophyletic clade. This indicates that the *Rp1* genes maintained in *B73* are more similar to each other than to *Rp1* genes in the other four maize lines. The same is true for the *Rp1* genes maintained in *HRp1-D*. Therefore, the *Rp1* genes in *HRp1-D* and B73 are evolving in a concerted manner. This type of evolutionary pattern was also observed when Ramakrishna *et. al.* (2002) sequenced 4 of the 15 *Rp1* genes from a different B73 haplotype and found that 2 of the genes differed by a single nucleotide change. It has been demonstrated that in most gene families, orthologs from different haplotypes are often more similar in sequence than are paralogs in the same

haplotype (Meyers *et al*. 1999) as is the case in this study for *HRp1-B*, *HRp1-M* and H95 but not for *HRp1-D* and B73. It is not apparent as to why some haplotypes show more evidence of within haplotype homogenization than others. Perhaps haplotypes similar to *Rp1-D* and B73 evolved from an extended period in the population with limited variation at the *Rp1* locus, while haplotypes like *HRp1-B*, *HRp1-M* and H95 evolved during a period of high genetic diversity.

Identical *Rp1* genes were not identified in *HRp1-B*, *HRp1-M*, B73 and H95 when compared. This in conjunction with the fact that most maize lines appear to have different *Rp1* haplotypes when compared by Southern blot analysis indicates that maize germplasm carries many hundreds of different *Rp1* genes with the potential to create new R-genes with new resistance specificities as a result of recombination and diversifying selection. Only a few of the *Rp1* haplotypes have been characterized in detail. Analysis of the haplotypic diversity of NB-LRR R-genes in previously uncharacterized *Rp1* haplotypes provides insights into *Rp1* R-gene number, transcription and diversity. This type of analysis is necessary to create a data resource for future use of this class of R-genes and to select the appropriate *Rp1* parental haplotypes used for crosses. This study characterized two *Rp1* haplotypes (*HRp1-B*, *HRp1-M*) differing in resistance specificity and two maize inbred lines (B73 and H95), that would be appropriate to generate the crosses needed for detailed resistance gene characterization, generation of recombinant haplotypes with new resistance specificities and improved maize cultivar performance.

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Table A.1 Unique *Rp1* genes and transcribed paralogs identified in five maize lines.

Maize line	Total clones analyzed	Unique *Rp1* genes: transcribed *Rp1* genes	*Rp1* paralog designations
HRp1-B	192	16:8	*Rp1-B-p1*, -p19*, -p24*, -p27*,-p28*, -p35*, -p36*, -p37*, -p60, -p61, -p62, -p63, -p64, -p65, -p66,* and *-p67*
HRp1-M	96	19:12	*Rp1-M-p1*, -p3*, -p4*, -p7*, -p8*, p10*, -p11*, -p14*, -p19*, -p20*, -p25*, -p30*, -p60, -p61, -p62, -p63, -p64, -p65,* and *-p66*
B73	96	14:4	*Rp1-B73-p3, -p5, -p6*, -p8, -p11, -p13, -p16, -p17*, -p18*, -p45*,-p61, -p67, -p71*,* and *-p73*
H95	160	12:8	*Rp1-H95-p1*, -p8*, -p12*,-p17*, -p28*, -p29*, -p32*, -p50*, -p60, -p61, -p62,* and *-p63*
[a]*HRp1-D*	-	9:7	*Rp1-D-p1*, -p2*, -p3*, -p*4, -p5, -p6, -p7*, -p8*,* and *-p9**
Total	544	70:39	

* Indicates transcribed *Rp1* paralogs. [a] Previously characterized *Rp1* haplotype.

Table A.2 Haplotype diversity in the C-terminal LRR region of *Rp1* genes in four maize lines.

Maize line	Region [a]	Sites [b]	S [c]	H [d]	H_d [e]	π [f]	Θ_w [g]
HRp1-B	4449-5155	707	342	10	0.983	2.28%	2.03%
HRp1-M	4512-5201	690	351	11	0.994	3.31%	2.00%
B73	4536-5150	615	52	10	0.989	.51%	.48%
H95	4443-5150	708	371	8	1.00	1.35%	2.09%

[a]Region represents the range of the LRR domain in the aligned dataset.
[b]Sites represents the number of nucleotides in the analyzed domain.
[c]*S* (segregating site) represents the number of segregating (polymorphic) sites (Nei 1987).
[d]*H* represents number of unique haplotypes (Nei 1987).
[e]$H_d = (1 - \Sigma xi\ 2)\ n / (n - 1)$, where xi is the frequency of a haplotype and n is the sample size.
π (Nucleotide diversity) represents the average number of nucleotide differences per site between two sequences (Nei 1987) .
[g]θ_w ($\theta = 4Nu$) for an autosomal gene of a diploid organism (N and u are the effective population size and the mutation rate per DNA sequence per generation, respectively) (Tajima 1983).

Table A.3 Recombination in the C-terminal LRR region of *Rp1* genes in four maize lines

Maize line	(*R*) events [a]	(*R*) between adjacent sites [b]	(*R*) per gene [c]
HRp1-B	19	0.0012	0.001
HRp1-M	22	0.0023	1.5
B73	3	0	0.001
H95	9	0	0.001

[a]*R* represents the minimum number of recombination events between sites (Hudson *et al*. 1987).

[b]*R* represents the estimate of recombination between adjacent sites calculated based on the average number of nucleotide differences between pairs of sequences (Hudson *et al*. 1987).

[c]*R* represents the estimate of recombination per gene (Hudson *et al*. 1987).

Figure A.1 Neighboring joining tree of *Rp1* genes in *HRp1-B.*

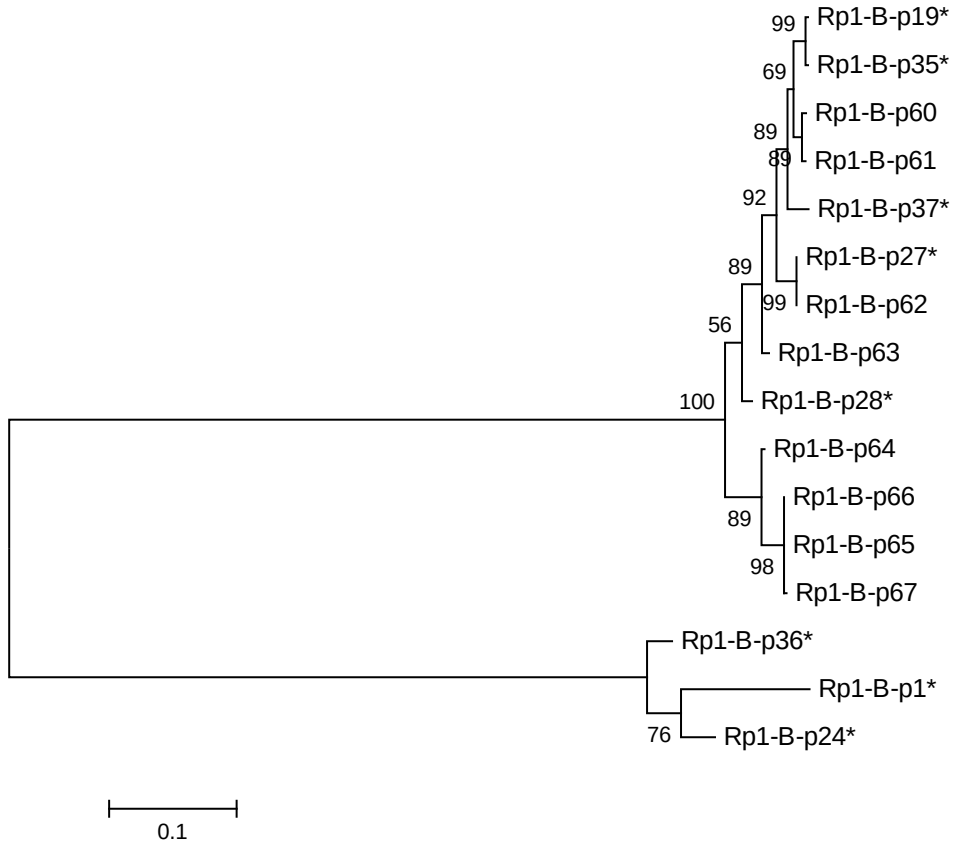


Figure A.2 Neighboring joining tree of *Rp1* genes in *HRp1-M*.

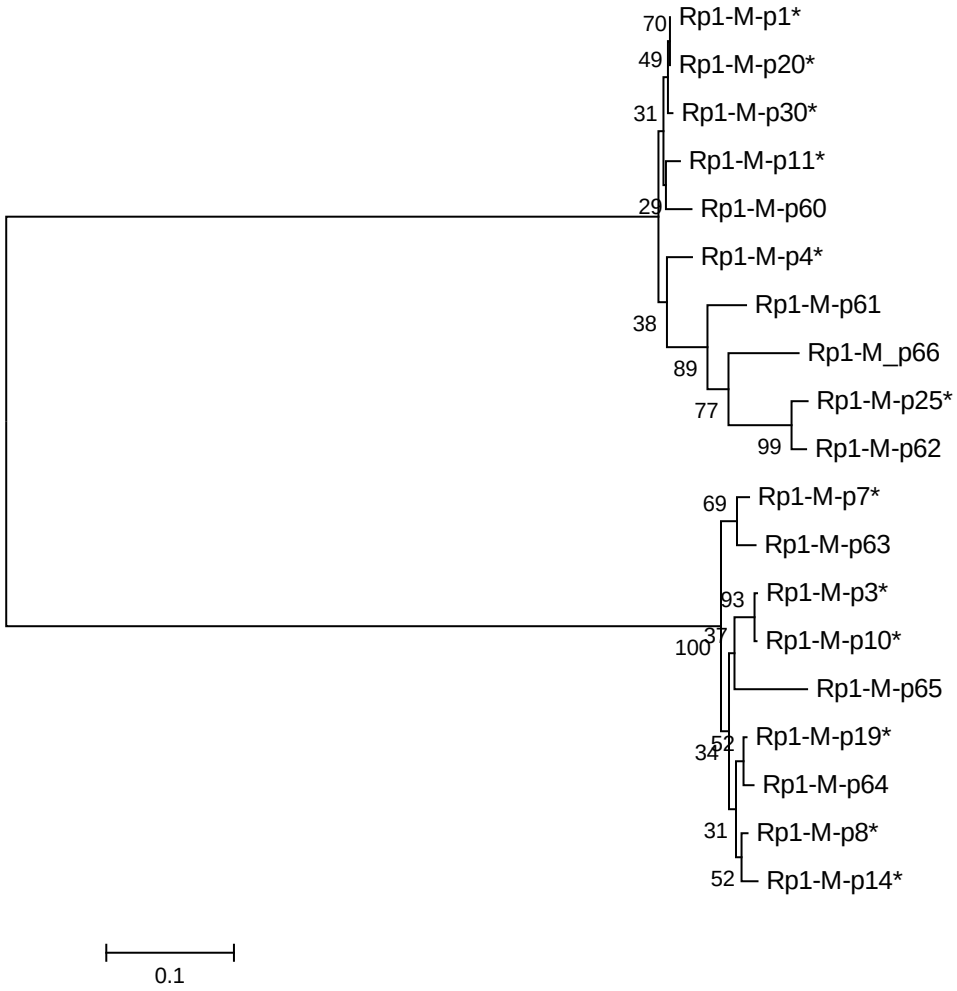


Figure A.3 Neighboring joining tree of *Rp1* genes in B73.

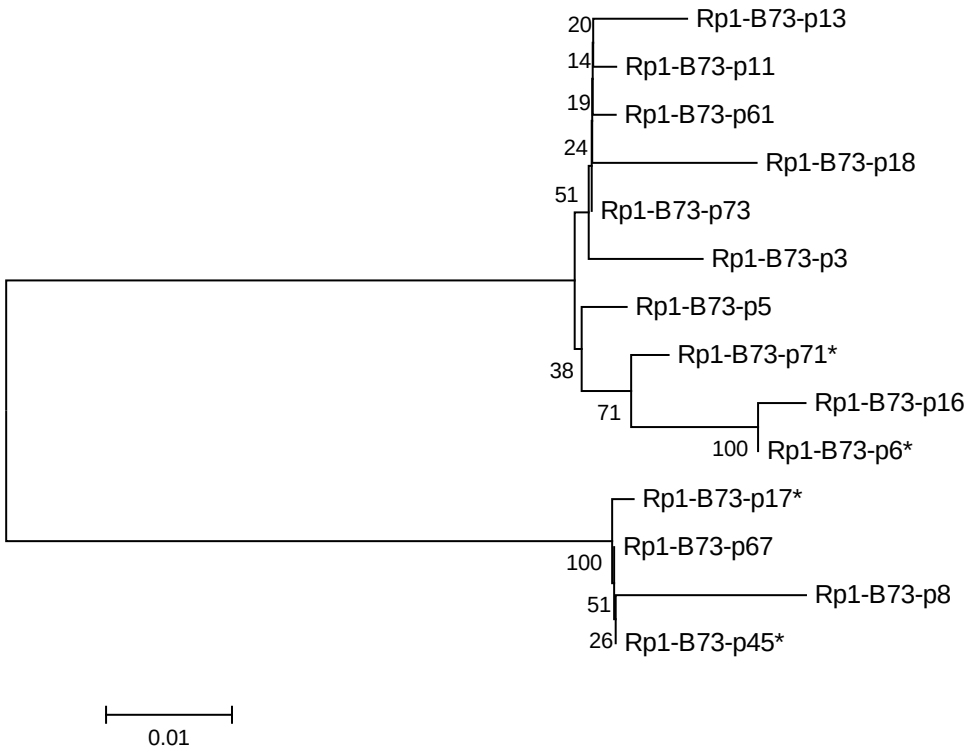


Figure A.4 Neighboring joining tree of *Rp1* genes in H95. *Rp1* indicates the locus analyzed.

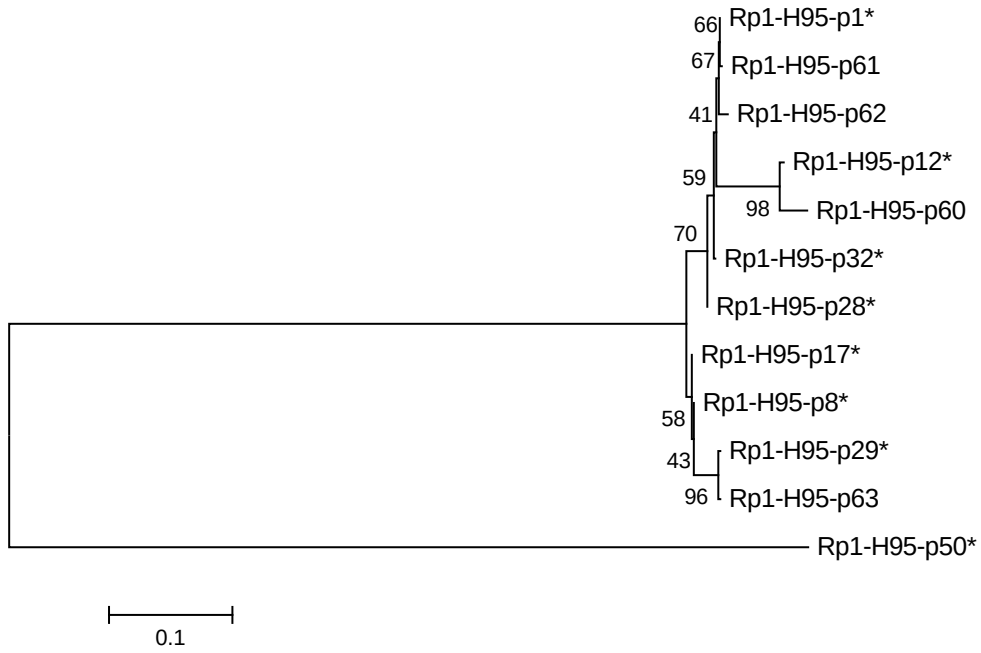
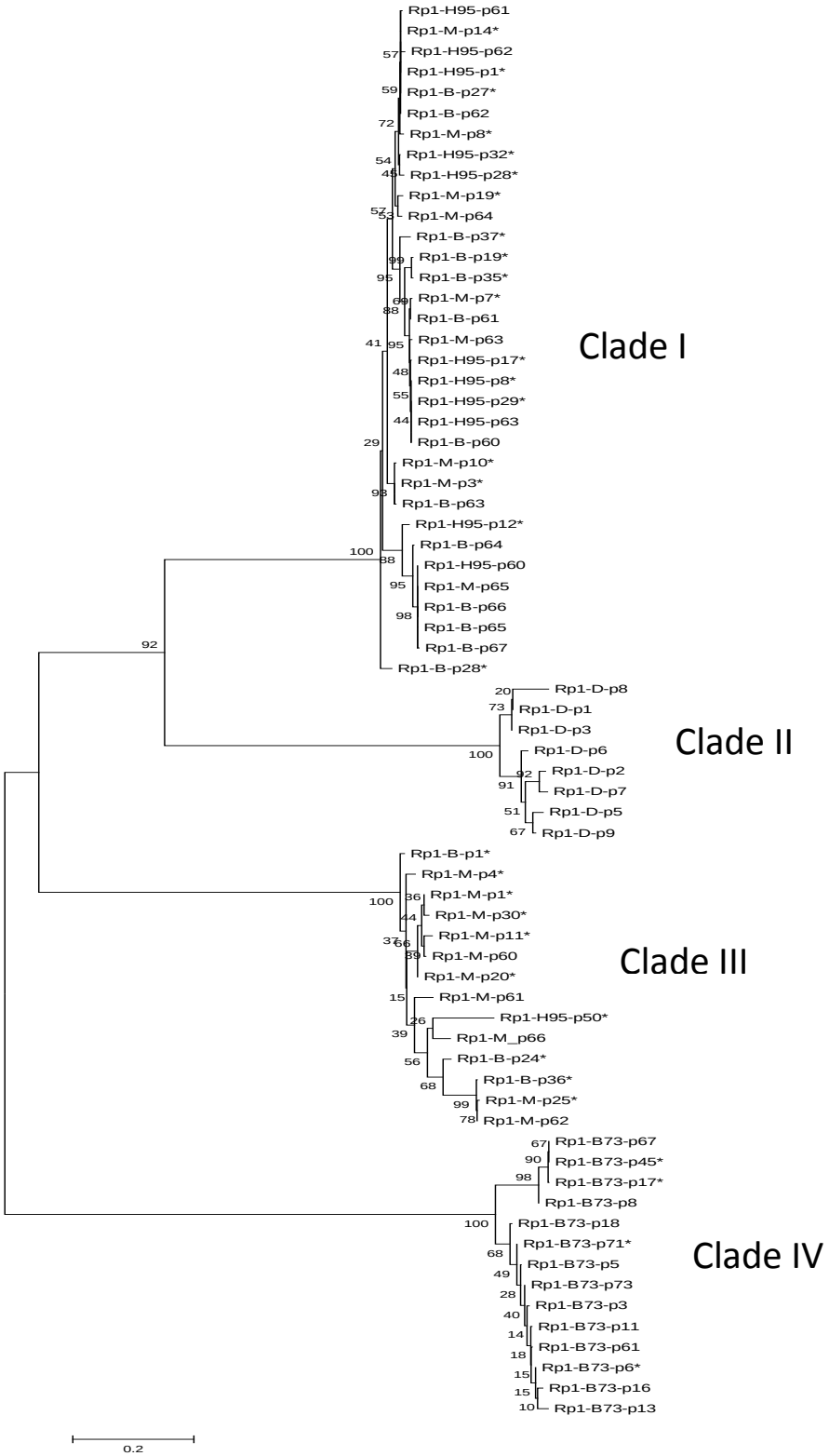


Figure A.5 Neighboring-Joining phylogenetic tree of *Rp1* genes isolated from *HRp1-B*, *HRp1-M*, B73 and H95.



APPENDIX B

A RAPID AND EFFICIENT METHOD FOR ASSESSING PATHOGENICITY OF *USTILAGO MAYDIS* ON MAIZE AND TEOSITE LINES[1]

[1] S. Chavan and S. M. Smith. *Journal of Visual Experimentation Published January 3, 2014.*

Abstract

Maize is a major cereal crop worldwide. However, susceptibility to biotrophic pathogens is the primary constraint to increasing productivity. *U. maydis* is a biotropic fungal pathogen and the causal agent of corn smut on maize. This disease is responsible for significant yield losses of approximately $1.0 billion annually in the U.S.[1] Several methods including crop rotation, fungicide application and seed treatments are currently used to control corn smut.[2] However, host resistance is the only practical method for managing corn smut. Identification of crop plants including maize, wheat and rice that are resistant to various biotrophic pathogens has significantly decreased yield losses annually.[3,4,5] Therefore, the use of a pathogen inoculation method that efficiently and reproducibly delivers the pathogen in between the plant leaves, would facilitate the rapid identification of maize lines that are resistant to *U. maydis*. As a first step toward indentifying maize lines that are resistant to *U. maydis*, a needle injection inoculation method and a resistance reaction screening method was utilized to inoculate maize, teosinte and maize x teosinte introgression lines with a *U. maydis* strain and to select resistant plants.

Maize, teosinte and maize x teosinte introgression lines, consisting of about 700 plants, were planted, inoculated with a strain of *U. maydis* and screened for resistance. The inoculation and screening methods successfully identified three teosinte lines resistant to *U. maydis*. Here a detailed needle injection inoculation and resistance reaction screening protocol for maize, teosinte and maize x teosinte introgression lines is presented. This study demonstrates that needle injection inoculation is an invaluable tool in agriculture that can efficiently deliver *U. maydis* in between the plant leaves and has provided plant lines that are

resistant to *U. maydis* that can now be combined and tested in breeding programs for improved disease resistance.

Keywords *Ustilago maydis*, needle injection inoculation, disease rating scale, plant-pathogen interactions.

Introduction

Fungal diseases of plants represent one of the most eminent threats to agriculture. The need to develop crops with improved disease resistance is increasing due to the food needs of a growing world population. Plant pathogens naturally infect crop plants in the field causing diseases that negatively impact crop yield.[6] It has been shown that identifying and utilizing resistant plants can improve resistance and decrease yield loss. Resistant cultivars have been identified in many plant species including maize, wheat, rice and sorghum by inoculating the plants with a plant pathogen and selecting for resistant lines.[7] Therefore, development and use of an efficient inoculation method would allow many plants to be inoculated and screened for resistance. Various inoculation methods have been used including dip inoculation, pipetting the pathogen cell suspension culture into the whirl of the plant and needle injection inoculation.[8-10, 11] With each method, the pathogen must reliably be introduced in between the plant leaves where the pathogen enters the plant through the formation of appresoria to ensure pathogen development and plant infection.[12,13]

The dip inoculation method involves submerging a plant seedling into a pathogen cell suspension culture, while the pipetting method requires placing the pathogen cell suspension culture into the whirl of the plant seedling. However, there are issues with both methods. First, both methods depend on the natural movement of the pathogen from the leaf surface into the plant tissue which is highly variable. Most pathogens naturally enter the plant through stomatal openings or wounds on the plant leaf surface. However, there is significant variability in the pathogens ability to penetrate the plant leaf surface through the stomata and/or wounds on the leaf surface. Therefore, pathogen penetration cannot be controlled with either inoculation method potentially resulting in inconsistent data. Second, when screening a

large number of plants, submerging the seedlings into a pathogen cell suspension culture can be time consuming and may limit the number of plants that can be screened. Conversely, the needle injection inoculation protocol described herein delivers the pathogen cell suspension culture in between the plant leaves facilitating the formation of appressoria.[14] The pathogen then utilizes the newly developed appressoria to enter the plant eliminating the pathogen penetration issue. Additionally, the needle injection inoculation protocol provides a range of phenotypes for maize and teosinte plants that have been inoculated with *U. maydis* and demonstrate good infection. The phenotypes can be used as a marker to determine the best concentration for the pathogen cell suspension culture resulting in consistent plant phenotypes within and between different experiments.

Following plant inoculation with a pathogen cell suspension culture, plants are typically screened to detect a resistant or susceptible phenotype.[8-10,11,15] While disease rating scales have being used extensively to screen and classify plant phenotypes, rating scales differ depending on the pathogen being analyzed. Therefore, a disease rating scale protocol establishment for *U. maydis* and maize interactions can be utilized for similar fungal pathogens.[16]

The present series of protocols details needle injection inoculation with a *U. maydis* cell suspension culture and disease resistance reaction screening of maize, teosinte and maize x teosinte introgression lines. The present protocols are not limited to needle injection inoculation of *U. maydis* into maize plants but can be utilized for relatively any fungal pathogen and plant species. Therefore, including the details of both methods in the same protocol will enable researchers to directly utilize the protocols for inoculation and screening or to manipulate the original protocols to better fit the pathogen and plant species of interest.

Materials and Methods

1. Growth of Plant Material

1.1) Select plant lines for inoculation and screening. Two maize lines, five teosinte lines and forty maize x teosinte lines with uncharacterized resistance to *U. maydis* were used for this work (Table B.1).

1.2) Plant seeds for experimental (*U. maydis* injection) and control (water injection) needle injection inoculation experiments. Do this for each plant line.

1.3) Plant four seeds (replicates) for each plant line in small flats by pushing the seeds about ½ inch into the soil with finger and covering with soil lightly **(**Figure B.1 and B.2**)**. Do not pack the soil over the seed. Planting the seed deeper or packing the soil over the seed may cause problems with seedling emergence.

1.4) Water the seeds into the soil. Ensure that the soil is soaked and the seeds remain under the soil after watering.

1.5) After watering, place plants in a growth chamber with day and night environments of 28/20°C temperature and 14/10 hour of photoperiod, respectively and approximately 500 μmol m^{-2} sec^{-1} photosynthetically active radiations at the top of the canopy. Maintain the relative humidity during the day and night at approximately 70% and 90%, respectively.

1.6) Keep all plants in the same growth chamber to maintain a growth environment that is congruent across the experiment.

1.7) After 10 days, remove the plants from the growth chamber and inoculate the plants with the *U. maydis* cell suspension culture using a needle injection inoculation method. Note: Maize plants can be inoculated 7 days after planting. [8-10] However, the teosinte plants are too

small after 7 days. Therefore, inoculate both maize and teosinte plants 10 days after planting for consistency within the experiment (see step 2.12).

2. Needle Injection Inoculation

2.1) Do all work in a laminar flow hood. Remove *U. maydis* glycerol stocks from freezer storage. Use a sterile loop and streak glycerol stocks of *U. maydis* wild-type strains ½ (mating type a1b1) and 2/9 (mating type a2b2, near isogenic to ½) on to potato dextrose agar (PDA) plates. Maintain strains separately.

2.2) Place PDA plates streaked with *U. maydis* in a 30°C incubator for two days. If using a different biotrophic pathogen use the appropriate strain, media and growth conditions. Monitor the growth of the pathogen over the two day period to ensure that the *U. maydis* strain is growing well.

2.3) Remove the PDA plates from the incubator after two days. The plates should have good pathogen growth and contain single colonies (Figure B.3). It is important to obtain single colonies. If single colonies are not present re-streak the plates at a lower concentration.

2.4) Do all of the work in a laminar flow hood. Use a sterile toothpick to select a single colony for each strain from the PDA plates. Place the toothpick containing a single colony into a 3 milliliter (mL) potato dextrose broth (PDB). It is advised to have two to three cultures.

2.5) Place the 3 mL PDB cultures into a 30°C incubator/shaker for two days at 200 rpm. Monitor the growth of the culture over the two day period to ensure growth of the culture. The culture should appear very cloudy.

2.6) Remove the liquid cultures from the incubator/shaker and measure the concentration at OD_{600} to ensure that the cells were grown to an OD of 1.0 (~1 x 10^7 cells/mL).[17]

2.7) Bring the *U. maydis* cell suspension cultures to a final concentration of 1 x 10^6 cells/mL, using water in a final 30 mL culture volume. This concentration consistently results in good infection of the plants with the pathogen cell suspension culture.[17]

Note: Various cell suspension concentrations should be tested when using different pathogen strains to determine the appropriate cell titer needed for inoculation.[18, 19] The given final concentration for the cell suspension culture can be used as a starting point for tittering. The appropriate concentration of the pathogen cell suspension culture should be verified by visualizing the plant phenotypes with good infection (Figure B.6 thru B.10).

2.8) Mix equal volumes of the two *U. maydis* strains prior to inoculation. If using one pathogen strain proceed to step 2.9. Prepare fresh *U. maydis* cell suspension cultures for each inoculation experiment and discard cell suspension cultures after two days.

2.9) For the experimental needle injection inoculation, fill a 3 mL syringe with the *U. maydis* cell suspension culture by drawing the cell suspension culture into the syringe.

2.10) For the control needle injection inoculation, fill a 3 mL syringe with water.[17] Use the same procedure for the experimental needle injection inoculation.

2.11) Attach a .457mm x 1.3cm hypodermic needle to the end of each 3 mL syringe. The selected needle size will deliver the cell suspension culture in between the plant leaves with minimal damage to the plant tissue.

2.12) Remove the experimental and control plants from the growth chamber 10 days after planting in preparation for needle injection inoculations (Figure B.4) (see step 1.7).

2.13) Carefully insert the hypodermic needle containing the *U. maydis* cell suspension culture into the stem of an experimental plant at a 90° angle just above the soil line. Insert the

needle until it is in the middle of the stem. Do not push the needle through the stem **(**Figure B.5).

2.14) Inject the experimental plant with about 100 µl of the *U. maydis* cell suspension culture.[18,19] This will vary slightly depending on the height of the seedling. The cell suspension culture will push through the stem and move into the whirl of the plant. The cell suspension culture will be visible in the whirl of the plant. Continue injecting 100 µl of the cell suspension culture into each individual plant until the 3 mL syringe is empty.

2.15) After the injection, carefully remove the needle from the plant stem. Remove the needle from the now empty 3 mL syringe and fill with water. Attach the needle back to the syringe and push the water through the needle to remove any plant tissue that may be caught in the needle tip.

2.16) Repeat steps 2.9 through 2.15 for each experimental plant. Follow the same protocol for the control plants by injecting water.

2.17) Place the inoculated experimental and control plants back into the growth chamber. Water the plants daily by wetting the soil not the plant tissue.

2.18) Check the plants daily to detect pathogen development and plant resistance reactions.

3. Resistance Reaction Screening

3.1) Score and record the resistance reactions for each plant 7, 10, 14 and 21 days post inoculation (dpi) using a 1 to 5 resistance reaction rating scale. Disease severity increases as the numerical values on the rating scale increases (Table B.2). A 1C (Leaf chlorosis), 1A (Leaf anthocyanin production) or 2 (small leaf galls) resistance reaction indicates resistance. A 3 (stem galls), 4 (basal gall) or 5 (plant death) resistance reaction indicates susceptibility (Figure B.3a, b, c, d, e and Table B.2).[18, 19]

3.2) Score both experimental and control plants and record resistance reaction ratings.

3.3) Compare the resistance reactions of the experimental and control plants. Select experimental plants with a 1C, 1A or 2 resistance reaction rating. These plants are considered to be resistant to *U. maydis*.[18, 19]

3.4) Repeat the entire experiment to verify the plant phenotypes.

Representative Results

A successful needle injection inoculation can be determined by visualizing the phenotype of the plants inoculated with *U. maydis* (experimental). The majority of the experimental plants were susceptible to *U. maydis* infection. The susceptible plants showed very severe disease development demonstrated by stem and basal gall formation with black teliospores (Figure B.9, B.10 and TableB. 2). Several plants were dead after inoculation due to the severity of the disease. Three maize x teosinte introgression lines that were resistant to *U. maydis* were identified. For plants resistant to *U. maydis*, a successful inoculation was demonstrated by minor chlorosis, anthocyanin production or, minor leaf gall formation. (Figure B.6,B.7,B.8 and TableB. 2).

To verify that the phenotype observed for the experimental plants was the result of the inoculation, the phenotypes of the experimental and control plants (water inoculated) were compared. The experimental plants showed pathogen development on the leaf and/or stem area as described above for the resistant and susceptible plants. Conversely, the control plants did not demonstrate a phenotype. The controls plants were very clean and did not show pathogen development on any part of the plant, indicating that pathogen development on the experimental plants was due to the needle injection inoculation with *U. maydis*.

To verify the reproducibility and efficiency of the needle injection inoculation method, the experiment was performed twice consisting of 700 plants and compared the resistance reaction scores (phenotypes) for the experimental plants within and between experiments for each plant line. The four replicate plants from the same plant line within one experiment showed the same resistance reaction score for 99.8% of the plants. Additionally, the four replicate plants were compared between experiments and indicated that 99.4% of the plants showed the same resistance reaction score. This suggests that the needle injection inoculation method can efficiently deliver the *U. maydis* cell suspension culture in between the plant leaves and that the inoculations and phenotypes were consistent within and between experiments.

Discussion

In this study the needle injection inoculation method used to deliver a strain of *U. maydis* into the stem of 700 maize and teosinte plants was successful. Additionally, a revised disease resistance rating scale was used to screen the plants and detect pathogen development. As a result of using both methods, plant lines that are resistant to *U. maydis* were identified among 700 maize and teosinte plants that can now be combined and tested in breeding programs for improved disease resistance.

As with most inoculation methods, the ability to reproduce the same resistance phenotype among plants from the same line is essential. Additionally, the same resistance phenotypes must be observed in at least two separate experiments.[20,21] Because the ability to obtain a plant phenotype, whether it is resistant or susceptible, is primarily determined by the ability of the pathogen to gain access into the plant tissue, it is very important to select an inoculation method that delivers the pathogen in between the plant leaves each inoculation. A

few of the common issues researchers have faced with needle injection inoculation methods using biotrophic fungal pathogens such as *U. maydis* are: 1) Inappropriate concentration of fungal pathogen used for inoculation, 2) lack of reproducible phenotypes in multiple experiments, and, 3) lack of an established resistance reaction scoring method. Here each of the issues is addressed separately.

It is important to determine the appropriate concentration of the fungal pathogen cell suspension culture used for inoculation.[8-10, 11, 22] Inoculation with high concentrations of the pathogen cell suspension culture will cause the death of both resistant and susceptible plants, while low concentrations will not show a phenotype on either plant type. However, the appropriate concentration of the fungal pathogen cell suspension culture used for inoculation will vary depending on several factors including the pathogen, pathogen strain, plant species and plant accession. The present protocols provide phenotypes and a concentration for the *U. maydis* cell suspension culture to be used as a starting point to test the titer for needle injection inoculation. This results in consistent plant phenotypes within and between different experiments. The cell suspension culture concentration used for *U. maydis* inoculations can also be used as a starting concentration for inoculations with other biotrophic fungal pathogens. It is advisable to test different dilutions of the pathogen cell suspension culture when using other biotrophic fungal pathogens. This will facilitate the selection of the best concentration for the pathogen cell suspension culture being used for inoculation.

A large number of plants typically must be inoculated and screened from a plant population to potentially identify plants resistant to the pathogen of interest.[6,23] Therefore, it is important to utilize an inoculation method that reliably delivers the pathogen cell suspension culture in between the plant leaves and that this is done with relative ease and

little manipulation of the plants. This will facilitate reproducible phenotypes in multiple experiments. The present protocols give a detailed outline of a needle injection inoculation in the stem of maize and teosinte plants with a *U. maydis* cell suspension culture. This method can also be used for inoculation of other plant species similar to maize and teosinte. In order to cause disease in the plant, *U. maydis* must move into the plant tissue.[7,21,24] During natural infection, *U. maydis* moves into the plant tissue through stomatal openings or wounds on the plant leaf surface. A dip inoculation and plant whirl pipetting method has also been used to mimic the *U. maydis* natural infection process but has had limited success due to the variability in the pathogens ability to penetrate the plant tissue.[8-10,25] However, the needle injection inoculation method delivers the *U. maydis* cell suspension culture in between the plant leaves eliminating the pathogen penetration issue.

Establishment of a resistance reaction rating scale for *U. maydis* in essential to identify plants resistant to the pathogen.[25] The present protocols give a detailed description of the 1 (resistant) to 5 (susceptible) disease rating scale established for *U. maydis* infection of maize and teosinte plants. It is impotent to first perform a test inoculation and screen a small number of plants prior to initiating a large scale experiment with hundreds of plants. The resistance reaction rating scale established in the present protocol demonstrated consist phenotypes for 700 plants in two different experiments. It is advised to repeat the inoculation and screening protocols at least twice to demonstrate consistency and reproducibility of the results.

The present needle injection inoculation method and the established resistant reaction rating scale will continue to be used to screen and select maize and/or teosinte plants that are resistant to *U. maydis* infection. As a result, the two methods have many important

implications in agriculture that can be used in breeding programs for improved resistance to *U. maydis* infection decreasing yield losses in the U.S. and internationally.

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Tables

Table B.1 Resistance responses of maize and teosinte lines inoculated with *U. maydis*.

Plant Lines	Plant Species	Resistance Response
Zea mays (NSL 30060)	Maize	Resistant
Zea mays subsp. mays (PI511562)	Teosinte	Susceptible
Zea mays subsp. parviglumis	Teosinte	Susceptible
Zea mays subsp. diploperennis	Teosinte	Resistant
Zea mays subsp. luxurians	Teosinte	Resistant
B73 (P1)	Maize	Susceptible
Zea mays subsp parviglumis (P2)	Teosinte	Susceptible
Z031E0560	Maize x Teosinte NIL	Resistant
Z031E0560	Maize x Teosinte NIL	Resistant
Z031E0068	Maize x Teosinte NIL	Resistant
37 maize x teosinte NILs	Maize x Teosinte NILs	Susceptible

P1 indicates parent of the NILs.
P2 indicates parent of the NILs.
NIL indicates Near-isogenic-lines.

Table B.2 Resistance reaction rating system used for *U. maydis* scoring.

Host Response	Disease Rating*	Disease Symptoms*
Resistant	1C	Few chlorotic areas, no gall formation.
Resistant	1A	Dark purple anthocyanin production, few galls formed.
Resistant	2	Minor leaf galls.
Susceptible	3	Severe stem galls with the formation of black teliospores.
Susceptible	4	Large basal galls with the formation of black teliospores
Susceptible	5	Death of plants with severe leaf, stem and basal galls.

*Rating and disease symptoms correspond to the phenotypes in figure 3.

Figures

Figure B.1Six maize seeds placed on top of soil for planting.

Figure B.2Maize seeds pushed ½ inch into the soil with finger.

Figure B.3 Growth of *U. maydis* streaked on PDA plates after two day incubation at 30°C.

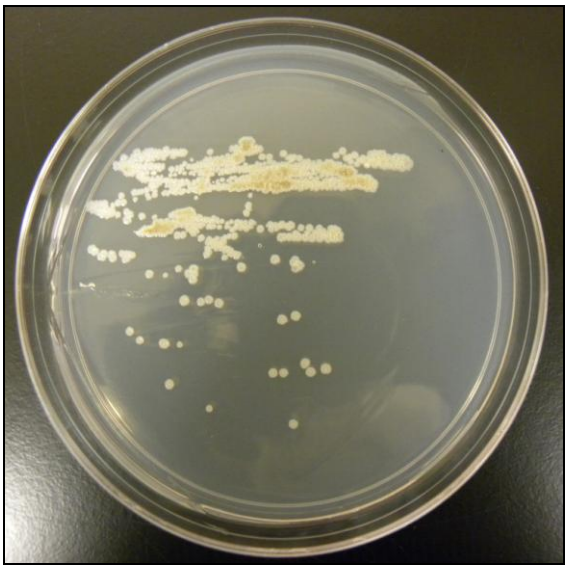

Figure B.4 Flat of uninoculated 10 day old maize seedlings removed from the growth chamber.

Figure B. 5 Needle injection inoculation in the stem of ten day old seedling with 100 µl of the *U. maydis* cell suspension culture

Figure B.6 Plant phenotypic responses to *U. maydis* needle injection inoculation. Resistant teosinte plants with minor leaf chlorosis exhibited by white streaks on the leaves.

Figure B.7 Plant phenotypic responses to *U. maydis* needle injection inoculation. Resistant teosinte plants with Anthocyanin production exhibited by the purple leaf color. The phenotype corresponds to a 1A resistance reaction rating score.

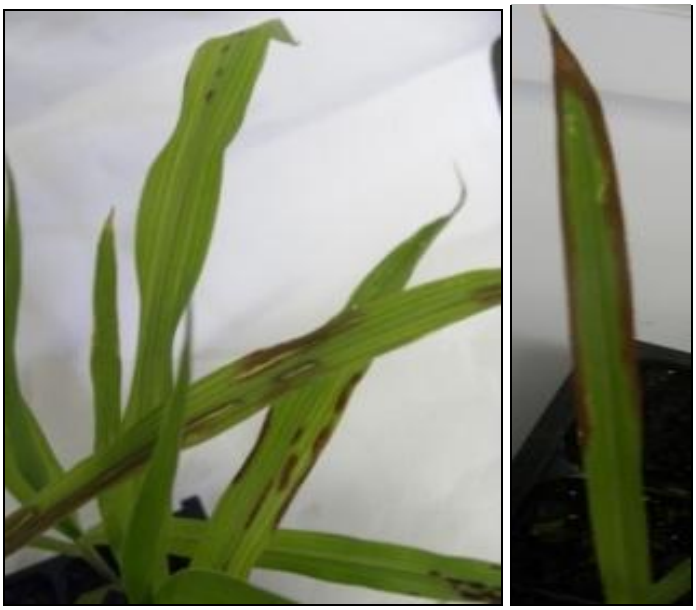

Figure B.8 Resistant teosinte plants with minor leaf gall development. The phenotype corresponds to a 2 resistance reaction rating score.

Figure 3.9 Susceptible maize plants with severe stem gall development and black teliospores. The phenotype corresponds to a 3 resistance reaction rating score.

Figure 3.10 Susceptible maize plants with severe basal gall development. The phenotype corresponds to a 4 resistance reaction rating score.