CHARACTERIZATION OF RESISTANCE TO *USTILAGO MAYDIS* IN TEOSINTE AND MAIZE-TEOSINTE INTROGRESSION LINES

by

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(Under the Direction of Shavannor M Smith)

ABSTRACT

Ustilago maydis, a fungal pathogen that causes corn smut disease, infects all aerial parts of maize plants and induces local tumors. The lack of *U. maydis*-resistant maize cultivars has led to significant corn yield losses. Therefore, it is important to identify new sources of resistance to *U. maydis*. Two maize-teosinte near-isogenic lines (NILs), resistant to *U. maydis* were identified from phenotypic evaluation of an introgression population created by crossing a maize inbred line (B73) and a teosinte (*Zea mays* ssp. *parviglumis*) inbred line (TIL11). Genotypic analysis identified a 3.9-Mbp teosinte introgressed region on the short arm of chromosome 9 that was present in the two resistant NILs but was absent in the susceptible NILs, suggesting the teosinte introgressed region was responsible for the resistant phenotype. Comparative analysis of the two parental lines and 25 Nested Association Mapping population founder lines identified 69 genes in the 3.9-Mbp region, with 24 genes classified as putative disease resistance, pathogenesis related or defense response genes. RNA sequence analysis of TIL11, B73, and the two NILs identified 24 differentially expressed genes (DEGs) from the 3.9-Mbp region that were also putative disease resistance, pathogenesis related or defense response genes. Four DEGs (1. PK_Tyr_Ser-Thr/Pkinase; 2. LTP_2/Tryp_alpha_amyl; 3. TPR_1; 4. no Pfam) were upregulated

in TIL11 and the two NILs and were selected as candidate genes for resistance. To investigate the role of the four genes in response to *U. maydis* infection, *Foxtail mosaic virus*-induced gene silencing was used to knock down the expression of each gene in TIL11 and the two NILs. The resistance of TIL11 and the two NILs was significantly reduced in the knock down plants and correlated with the reduced relative expression observed for the four genes. These results indicate that the four candidate genes identified in the 3.9-Mbp region may be positively regulating resistance to *U. maydis* in the resistant TIL11 parent and the two resistant NILs, identifying teosinte as a source of resistance. This work provides new insight into the genes involved in resistance and will open the door for targeted strategies to generate maize lines with enhanced resistance to *U. maydis*.

INDEX WORDS: *Ustilago maydis*, Corn smut, Maize-teosinte introgression lines, *Zea mays* ssp. *parviglumis*, Near isogenic lines, Nested Association Mapping population founder lines, Comparative genome analysis, RNA sequencing, qRT-PCR, Virus induced gene silencing.

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DEDICATION

“To my dear mother, Lalita Bhatta. Mom, it has been nearly two decades since I lost you, but your influence on my academic journey and my personal growth has only grown stronger with time. Your unwavering support, hard work, dedication to education, sacrifices, love, wisdom, inspiration, motivation, and belief in my potential have been the driving force behind my achievements. I miss you dearly and love you more than words can express. I cherish the memories of our time together and hold dear the foundation you provided me. I dedicate this dissertation to you with all my heart, in honor of the legacy you left behind, the love that continues to guide me, and the profound impact you continue to have on my life. Wherever you are, I hope you know how much you mean to me, and I will always treasure the love and support you gave me.”

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

BACKGROUND AND PURPOSE OF THE STUDY

Ustilago maydis is a fungal pathogen that causes considerable yield losses in corn crops, estimated at around $1 billion annually in the United States alone. This pathogen is responsible for forming major leaf, stem, and basal galls, eventually leading to corn smut, and rendering the corn unmarketable. Cultivated maize is susceptible to *U. maydis*, making it a significant problem, particularly in developing countries. Although there are several management practices such as crop rotation, sanitation, chemical fertilizers, and certified seeds, host resistance is the most durable and environmentally friendly method for managing corn smut. The lack of durable resistant maize cultivars necessitates new sources of resistance to *U. maydis*. Teosinte, a wild ancestor of maize, has been shown to provide resistance to several pathogens and may be a potential source of resistance for *U. maydis* (Wei et al. 2003).

Well-characterized germplasm that can be used for improving plant genotypes is often limited in genetic diversity and can lead to the breakdown of disease resistance over time (Migicovsky and Myles 2017). Wild relatives are a largely untapped source of desirable traits such as disease resistance. Expanding the source of resistance to include wild maize relatives (teosinte) can provide a new source of resistance genes for introgression into maize cultivars. Additionally, the resistance genes from wild maize relatives have not been exposed to the current pathogen population in monoculture, and therefore have the potential to be durable. For this

study, *Zea mays* ssp. *parviglumis* (TIL11), the immediate progenitor of maize, was utilized as a potential source of resistance to *U. maydis*.

A broad understanding of the resistance and the defense-related genes present in a maize wild relative will provide a foundation for improving the resistance of commercial maize varieties to *U. maydis*. This knowledge would be useful in breeding programs as it facilitates the selection of specific plant genes from a maize wild relative that confer resistance to *U. maydis* and can be introgressed into maize lines for improved resistance to *U. maydis*.

Additionally, it will provide insight into the resistance mechanism that plants use against pathogens, which can be applied when creating new strategies to protect crops from fungal plant diseases. The present study aims to identify a new source of resistance to *U. maydis* and determine whether teosinte could be a source of effective resistance genes to corn smut.

LITERATURE REVIEW

Plant Pathogen Coevolution

Zhou et al. (2001) highlighted the ongoing arms race between parasites and their hosts in nature, which suggests that resistance is always transient. The growth of crops in areas far from their origins, where they lack coevolved pathogens, has led to a narrow genetic base and increased susceptibility to plant disease, threatening global food security. Pathogens like fungi, bacteria, viruses, oomycetes, nematodes, and parasitic plants can cause significant yield losses in the field and post-harvest. To manage plant diseases, integrated methods such as quarantine, exclusion, rotation, intercropping, and chemical control are employed, but the use of disease-resistant varieties is the most cost-effective and eco-friendly approach.

One promising method to achieve host plant resistance is to use plant disease resistance genes in breeding programs. However, cultivated species have limited sources of resistance, making it a challenge. To address this issue, wild progenitor species were introduced into breeding programs in the 1920s to provide resistance to pests, diseases, and abiotic stressors, and to improve crop performance (Loskutov 1999). These progenitor species have provided plant breeders with a broad pool of genetic resources that have been used to enhance modern agriculture (Rick and Chetelat 1995; Hoisington et al. 1999; Suszkiw 2005; Hajjar and Hodgkin 2007). The use of wild species to provide resistance has increased significantly over the past decade, including teosinte, the wild ancestor of maize, which has shown resistance to various diseases and insects that could be durable. However, there have been limited reports of the use of teosinte to enhance maize resistance against *U. maydis*, the causative agent of corn smut disease (Prescott-Allen 1988; Hajjar and Hodgkin 2007).

Resistance and Pathogenesis Related Genes

Plants use several different types of disease-resistance genes to detect the presence of pathogens and induce defense responses. Resistance genes (R-genes) mediated defenses are typically detectable in plant-pathogen interactions (2-3 hours) after delivery of effectors into the host cytoplasm (Abramovitch et al. 2006). Activation of plant defenses is initiated in a resistant reaction when the plant's R-genes recognize and interact (directly or indirectly) with its corresponding avirulence (AVR) proteins from the invading pathogen. These AVR proteins are called elicitors. The AVR gene products are effector molecules that interfere with plant defenses to trigger susceptibility. They are highly host specific and serve as ligands for the receptor molecules which are encoded by the R-genes (Flor et al. 1971). The interaction of the plant R-genes and the AVR genes of the pathogen is highly complex, which leads to the activation of

various signaling pathways that activate the defense response of the plants. Each R-gene in the host has a corresponding AVR gene in the pathogen, and only the corresponding AVR gene can initiate a hypersensitive response (HR), leading to incompatibility (Flor et al. 1971). Hypersensitive response is the initial response of the plant that causes apoptosis of the plant cells at the site of pathogen invasion. If one of the two genes (R-genes, AVR genes) is absent, the normal pathogenicity of the pathogen results in a compatible reaction resulting in disease. The R-gene and AVR genes are inherited dominantly and are essential for overcoming pathogen infection. In contrast, susceptibility and virulence are inherited in a recessive manner. Pyramiding multiple genetic resistance loci can achieve durability in a single elite cultivar.

There are five major classes of R-genes based on the structural characteristics of their protein product. The first class includes a majority of R-genes known as the nucleotide binding-site leucine rich repeat (NBS-LRR) resistance genes (Meyers et al. 2005; van Ooijen et al. 2008). NBS-LRR genes are abundant in plants. It estimated that at least 200 different NBS-LRR genes exist in Arabidopsis, representing 1% of its genome (Ellis et al. 2000). The second class includes R-genes coding for receptor-like proteins with a transmembrane domain and an extracellular LRR domain. Members of this class include *Cf-2* gene products mainly found in solanaceous species and are specific for leaf mold resistane and *HS1* specific for nematode resistance (Tameling and Takken 2008). The *Cf-2* gene product belongs to a class of proteins called receptor-like proteins (RLPs) and is involved in the plant's immune response to the pathogen *HS1* is a small, cysteine-rich protein that is secreted by the plant roots and accumulates in the nematode feeding sites, where it disrupts the nematode's feeding behavior and reduces its reproduction. The third class of R-genes codes for receptor-like kinases with an extracellular LRR, a membrane-spanning region and an intracellular protein kinase domain. This class is

exemplified by *Xa21*, *Xa26* and *Pi-d2* in rice (Ellis et al. 2000; Meyers et al. 2005). The fourth-class codes for serine/threonine kinase with the *Pto* gene among other genes. It confers resistance to tomato bacterial speck and encodes a serine/threonine protein kinase with no leucine-rich repeat (Ellis et al. 2000).

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 pathogenesis related proteins (PR proteins) (Maleck et al. 2000; Campos et al. 2002). Tissues distal to the initial site of infection experience an increased accumulation of several defense signals (Fu and Dong 2013). Subsequently, PR proteins, the systemic production of pathogen-induced antimicrobial proteins, enhance resistance to various pathogens (Durrant and Dong 2004). Induction of PR proteins is one of the major biochemical and molecular events that occur in various plant tissue due to the pathogens such as fungi, viruses, bacteria, and viroids (van Loon 1997). Tissues distal to the initial site of infection experience an increased accumulation of several defense signals (Fu and Dong 2013). The signaling pathway elements, including different receptor components or chemical elicitors such as salicylic acid, ethylene, jasmonic acid and systemin, induce PR proteins (Ward et al. 1991; Xu et al. 1994; Maleck et al. 2000; Campos et al. 2002). Pathogenesis related proteins are thought to play an important role in induced resistance *i.e.*, systemic acquired resistance. Some of these PR proteins show antifungal or antimicrobial activity and have a known function, such as chitinase or glucanase, whereas many have no assigned function (van Loon et al. 1994). However, susceptibility is correlated with very low or undetectable levels of PR gene expression. This indicates that activation of PR genes is directly associated with resistance.

Characterizing the expression levels of the R-genes, PR proteins, and other genes associated with defense function in teosinte and two maize-teosinte NILs in response to *U. maydis* infection will provide insight into the genes that contribute to resistance.

Origin and Economic Importance of Maize and Teosinte

Maize (*Zea mays* ssp. *mays*), also known as corn, is grown worldwide and is the most significant staple crop in many countries. It is primarily used for Food, Feed, Fiber and Fuel (4F) purposes, although a substantial amount is used for animal feed and biofuel production (Cassidy et al. 2013). Maize is a model system for studying genome structure, function, heritability and diseases affecting cereals and monocots (Chandler et al. 2002).

The United States is the world's largest corn producer, growing 375.4 million metric tons annually, accounting for 35% of global maize production. It is the most abundantly grown cereal, with an annual production of 1, 068.3 million metric tons from a total area of 185 million hectares (FAO 2021). The US plays a significant role in the world corn trade market, exporting around 19% of its annual production and accounting for $9.2 billion in total export value in 2020 (USDA 2020).

Maize belongs to the *Poaceae* family and originated from its wild ancestor, teosinte (*Zea* species). Genetic and archaeological data indicate that maize was domesticated 9,000 years ago from *Z. mays* ssp. *parviglumis* in the lowlands of Central Balsas, Mexico (Matsuoka et al. 2002; Piperno et al. 2009; van Heerwaarden et al. 2011). Balsas teosinte/TIL11 (*Z. mays* ssp. *parviglumis*) is the direct ancestor of maize. The multi-grained ears of maize are the result of human selection (Iltis 2000). Maize is reported to have a unidirectional gene flow and has introgressions of teosinte alleles into a maize background (Fukunaga et al. 2005). Domestication and artificial selection of maize have increased its quality and yield. These two processes have

also had a negative impact on maize resistance to pathogens and insects (Rosenthal and Dirzo 1997; Chen et al. 2015). Maize is typically planted in a monoculture over large acreages, contributing to the breakdown of resistance to many important fungal pathogens. *U. maydis* is one of the major fungal pathogens that infect maize.

Teosinte is the common name for the wild taxa of *Zea*. In the past, all teosinte lines were placed in the same species as the taxonomy emphasized plant morphology. This division was considered inappropriate since it resulted from human selection during maize domestication. The genus *Zea* is further divided into two sections, *Luxuriantes* and *Zea Luxuriantes* is comprised of three species (1. *Z. diploperennis*, 2. *Z. perennis*, and 3. *Z. luxurians*) and four subspecies (1. *Z. mays* ssp. *huehuetenangensis*, 2. *Z. mays* ssp. *parviglumis*, 3. *Z. mays* ssp. *mexicana*, and 4. *Z. mays* ssp. *mays*,). *Z. mays* ssp. *parviglumis* is the immediate progenitor of maize. TIL11 is a representative inbred line of the lowland teosinte *parviglumis* subspecies, which is phenotypically different from maize (Hufford et al. 2012). In the seedling growth stage, maize and teosinte look similar. However, the morphological differences are very prominent at maturity, especially in the inflorescence. Maize has a single stalk terminating in a tassel and two to five ears on the stalk. The ears of maize consist of numerous naked yellow to white colored kernels arranged in multiple rows on a single cob. In contrast, teosinte is highly branched, with each branch terminating in a long tassel and cobs containing 5-10 kernels, each enclosed in a hard fruit case (Matsuko et al. 2002). Teosinte has a brittle cob, while maize forms solid cobs that do not release their seeds.

Ustilago maydis and its Life Cycle

U. maydis is a member of the fungal phylum Basidiomycota is a biotrophic and dimorphic fungus that causes corn smut disease. Corn smut was first reported in Bathurst, New

South Wales, Australia, in 1911. This disease is characterized by chlorosis, anthocyanin production, stunting and the development of tumors on the leaves, stems, tassels and ears resulting in significant economic loss (Banuett 1995). It has a very narrow host range and only infects maize and teosinte lines (*Z. mays* ssp. *mexicana*). Iltis (1987) observed *U. maydis* on teosinte tassels that turned spikelets into diseased grains (sex change) via the production of gibberellins (a growth hormone). The economically significant outbreaks often result in asynchronous timing of silk emergence and pollen production. Pataky and Snetselaar (2006) reported 10% yield losses annually due to corn smut. As maize is considered a staple crop in the United States, a yield loss of 1% is equal to $189 million per year (Martinez-Espinoza et al. 2002). Corn smut was also reported to cause 1.7 million bushels yield loss in twenty-four U.S. corn-producing states and Ontario in 2016 (Crop protection network 2017). Although corn smut affects all types of corn, it has a greater economic consequence in sweet corn than in field corn.

U. maydis is considered a model organism for studying plant-pathogen interactions (Martinez-Espinoza et al. 2002). It allows microbiological techniques, the ability to mutate genes, DNA recombination, and genomics and the ability to complete its life cycle in a short time. This fungus has a genome size of 20.5 million bp, which is smaller than other pathogenic fungi. There are two distinct morphological and developmental forms in which *U. maydis* exists. One stage is the nonpathogenic unicellular, saprobic haploid budding yeast. The other stage is a filamentous dikaryotic generated by the fusion of two compatible haploid cells. The filamentous dikaryon enters the host plant where karyogamy, cell proliferation and spore formation take place (Banuett 1991). The activation of mitogen-activated protein kinase (MAPK) signaling cascade and a cyclic AMP (cAMP) pathway leads to morphological changes (D'Souza and Heitman 2001). These pathways are considered to regulate the transition from budding to

filamentous growth by transducing signals such as the availability of nutrients and lipids, acidic pH, and pheromones from the mating type (Klose et al. 2004). The fungal life cycle revolves around two loci, termed "a" and "b". The mating between strains that differ at these two unlinked loci results in the formation of an infectious dikaryon. The "a" locus is biallelic and possesses the pheromone-based recognition system that regulates the cell fusion event (Bolker et al. 1992). It consists of precursors (mfa1 and mfa2) and receptors (pra1 and pra2) of lipopeptide pheromones (Banuett 1995). The "b" locus is multiallelic and heterozygosity in dikaryons is required for disease development. The locus encodes a pair of homeodomain proteins, bE and bW, and are functional only as heterodimeric transcription factors with subunits derived from different alleles (Gillissen et al. 1992).

The fungus overwinters as teliospores in crop debris and in the soil, where it can remain viable for several years. Under favorable conditions, these teliospores germinate to produce specialized diploid spores **(Figure 1.1**). Meiosis occurs and haploid sporidia are produced by successive budding from probasidia. The basidiospores are carried by air currents or are splashed by water to young, developing tissues of corn plants. Basidiospores germinate and produce a hypha, which can enter epidermal cells directly. After the initial development, its growth stops, and the hypha usually wither and sometimes dies, unless it contacts and fuses with a haploid hypha derived from a basidiospore of the compatible mating type. The fusion results in the formation of a dikaryotic hyphae. The filament produces an appressorial peg that enlarges in diameter and hyphae grow into the plant tissues mostly intracellularly. Plant cells surrounding the hypha enlarge and divide to form galls. Galls are obvious within 10-14 days after infection. Localized infection occasionally occurs in young seedlings and local infections in older plants. The mycelium in galls remains intercellular during most of the gall formation but before

sporulation, the enlarged corn cells are invaded by the mycelium, collapse, and die. The dikaryon proliferates and differentiates inside the tumor resulting in production of melanized teliospores (Banuett and Herskowitz 1996).

Management of *Ustilago maydis*

Various management methods have been suggested for the corn smut, including crop rotation, sanitation, seed treatments, foliar fungicides, fertility modifications, and biological controls. Host resistance is the only effective, economical, environmentally friendly, and practical method for the long-term management of this disease.

A comparative study of maize and teosinte suggests that some teosinte species have valuable resistance to fungal pathogens and viruses of maize. More than 80% of the beneficial traits obtained from wild relatives are related to pest and disease resistance. For instance, *Z. mays* ssp. *parviglumis* has been found to be resistant to *Colletotrichum graminicola* (de Lange 2014). In addition, Findley et al. (1982) successfully introduced resistance to maize chlorotic dwarf virus (MCDV) by introgressing resistance from *Z. diploperennis* into maize. Disease resistance has also been transferred from *Z. mays* ssp. *mexicana* to maize for many diseases, such as maize stalk rot, maize rough dwarf disease, and maize chlorotic dwarf virus. Crosses between maize and teosinte have also been shown to increase resistance to certain pathogens (Barry et al. 1992; Wang et al. 2008). Despite the broad-spectrum resistance to several pathogens that teosinte species offer, their potential for employable resistant traits against *U. maydis* in modern maize is yet to be fully explored.

Comparative Genome Analysis

A complete and well-annotated genome sequence is an essential resource for genomic approaches. Whole genome sequencing has been instrumental in identifying a wide spectrum of genetic variation, as demonstrated by Golicz et al. (2016), and has led to improvements in the precision and efficiency of predicting phenotypes from genotypes.

Comparative genomics, which involves comparing the complete genetic material of one organism to that of another, is a powerful tool for understanding how species have evolved and for determining the function of genes and noncoding regions. This includes comparing gene number, gene content, and gene location, as well as the length and number of coding regions within genes, the amount of noncoding DNA in each genome, and conserved regions that are maintained in both prokaryotic and eukaryotic organisms (Golicz et al. 2016).

In a study by Huang et al. (2005), genomic information from tomato was used to clone *R3a*, a gene that confers race-specific resistance to *Phytophthora infestans*. *R3a* is a member of the *R3* complex locus on chromosome 11. Comparative analyses of the *R3* complex locus with the corresponding *I2* complex locus in tomatoes suggest that this is an ancient locus involved in plant innate immunity against oomycete and fungal pathogens. Similarly, comparative genomics plays a very important role in under-utilized crops like finger millet. The available full sequence information of rice made it possible to identify the genes influencing blast resistance in finger millet through comparative genomics (Babu et al. 2015).

Comparative genomics can also be used to trace evolutionary relationships between organisms and to identify differences and similarities within and between species (Sivashankari and Shanmughavel 2007). According to Hufford et al. (2012), a comprehensive assessment of the evolution of modern maize based on the genome-wide resequencing of 75 wild, landrace and

improved maizes lines found evidence of recovery of diversity after domestication that was likely to be introgression from wild relatives.

Comparative genetic mapping can be used to assess the degree of genome-wide colinearity in species with different genome sizes and reveal large-scale rearrangements, duplications, and deletions. For example, Gale and Devos (1998) used comparative mapping to show that genome colinearity persists across vast evolutionary distances in grass genomes and that differences between species result more from the expansion of intergenic regions than chromosomal rearrangements. In addition, Wu et al. (2019) demonstrated that the comparative genomic approach, which takes advantage of the genomic synteny among wheat and other cereal crops, is effective for developing molecular markers for the fine mapping of disease-resistance genes in wheat.

The genome sequence of teosinte can be a valuable resource for studying the genetic differences between B73 and TIL11. By comparing the genomes of these two plants, we can identify specific genes or regions responsible for important traits, such as resistance to *U. maydis*. However, it is unclear whether these genes are expressed in TIL11 and their role in disease resistance can be determined through comparative analysis. To fill this gap, further research, such as RNA sequencing and functional analysis, is needed to confirm the expression of these genes in TIL11 and their role in disease resistance.

RNA Sequencing and Functional Analysis

RNA sequencing (RNA-seq) is a powerful technique that allows for the comprehensive analysis of the transcriptome, the set of all RNA molecules in a cell or tissue. According to Wang et al. (2009), RNA-seq provides a high-throughput and quantitative measurement of gene expression, as well as the detection of novel transcripts and alternative splicing events. It has

become a widely used tool in various fields of research, including genetics, genomics, functional genomics, and epigenetics. In recent years, RNA-seq has been widely adopted in plant biology research to discover new genes, identify novel splice variants, and characterize gene expression patterns in response to various biotic and abiotic stress. For example, RNA-seq has been used to study gene expression in various plant species, such as rice (Thomas et al. 2018), soybean (Wang et al. 2019), arabidopsis (Park et al. 2019), maize (Sekhon et al. 2013) and peanut (Rathod et al. 2020), however, the sheer amount of data generated by RNA-seq can be overwhelming and requires downstream analysis to extract meaningful biological insights. According to Trapnell et al. (2012) and Li and Dewey (2011), downstream analysis involves a series of computational steps to process and analyze the raw RNA-seq data, such as reading alignment and quantifying gene expression, and functional annotation. An example of this is a study by Rathod et al. (2020), where differential expression analyses were performed in a susceptible peanut genotype JL-24 and a resistant genotype GPBD-4. The differentially expressed genes that were uniquely upregulated in resistant genotypes included PR proteins, thaumatin, and ethylene-responsive factors. In addition, genes such as chitinase and R genes such as NBS-LRR were found to be highly upregulated in the resistant genotypes, suggesting that these upregulated genes are involved in plant defense mechanisms.

RNA-seq and downstream analysis of TIL11, B73, and two NILs allow us to generate meaningful biological insights from the raw RNA-seq data, such as differential gene expression. However, RNA-seq does not provide information about the function of the genes identified. One way to fill this gap is through virus-induced gene silencing (VIGS), a technique that uses viral vectors to knock down the expression of specific genes to study their function. This approach can provide important functional information that complements the data obtained from RNA-seq.

Virus-Induced Gene Silencing

Virus-induced gene silencing (VIGS) is a powerful reverse genetics technique that allows for the functional characterization of genes in plants. According to Ding (2000), VIGS is based on the plant's endogenous RNA defense response that recognizes the accumulation of foreign double-stranded RNAs (dsRNA) and targets these sequences for degradation. Plant viruses can be engineered to carry sequences that direct silencing of target host genes, expression of heterologous proteins, or edit host genes (Mei et al. 2019).

Many plant virus vectors have been developed for transient gene expression or silencing in plants (Pignatta et al. 2007; Igarashi et al. 2009; Jupin 2013; Li and Yoshikawa 2015). One of the most widely used viral vectors for VIGS in plants is the *Foxtail mosaic virus* (FoMV). A study by Mei et al. (2016) showed that the FoMV infectious clone could establish a systemic infection in maize inbred lines, sorghum, and green foxtail, indicating the potentially wide applications of this viral vector system for functional genomics studies in maize and other monocots.

Furthermore, direct agro inoculation of maize seedlings by injecting 2-3mm above the coleoptiler node with a FoMV silencing construct has been successful in several maize genotypes (Beernink et al. 2021). Mei et al. (2016) showed that four genes, phytoene desaturase (functions in carotenoid biosynthesis), lesion mimic22 (encodes a key enzyme of the porphyrin pathway), iojap (functions in plastid development), and brown midrib3 (caffeic acid O-methyltransferase) were silenced and characterized in the sweet corn line Golden 3 Bantam.

The candidate genes that are differentially expressed in TIL11 and two NILs were targeted for gene silencing using the FoMV-mediated VIGS system. This may be an effective tool for transiently silencing genes, particularly R-genes and PR genes, in TIL11 and NILs to

determine the function of specific genes contributing to resistance against *U. maydis*. It can provide a deeper understanding of the phenotype of the TIL11 and NILs after silencing the expression of a particular gene allowing inference to its function. Additionally, the use of VIGS to study gene function has the potential to impact crop improvement by identifying genes that play a role in important agronomic traits, such as disease resistance and stress.

HYPOTHESIS AND OBJECTIVES

The main focus of this dissertation is to conduct a comprehensive analysis of the mechanism of *U. maydis* resistance in Teosinte and NILs by utilizing comparative genome analysis, RNA-sequencing, and virus-inducing gene silencing methods. This research contributes to creating comprehensive genetic and genomic resources for studying *U. maydis* resistance in Teosinte and NILs. Until now, the progress towards these objectives has been hindered by the lack of a fully annotated teosinte genome and 25 NAM founder lines. The study aims to identify key genetic factors contributing to *U. maydis* resistance, specifically focusing on R-genes, PR genes, and other defense-related genes. This is crucial for developing sustainable and effective management strategies to combat *U. maydis*, a pathogen that poses a significant threat to maize production.

The hypothesis of this study was that genes characterized from the teosinte and two resistant near-isogenic lines could be used as a source for improving resistance in modern, annual, temperate maize against *U. maydis*.

To test this hypothesis, I propose the following three objectives:

1. Identify candidate genes in a 3.9 Mbp region from teosinte putatively associated with resistance to *Ustilago maydis* in a maize-teosinte near-isogenic lines (NILs).

2. Select candidate genes in a 3.9 Mbp region from teosinte differentially expressed in two resistant maize-teosinte NILs and two parental lines in response to *Ustilago maydis* infection.
3. Validate the function of four differentially expressed genes putatively associated with resistance to *Ustilago maydis* in two maize-teosinte NILs and a teosinte parental line with virus-induced gene silencing.

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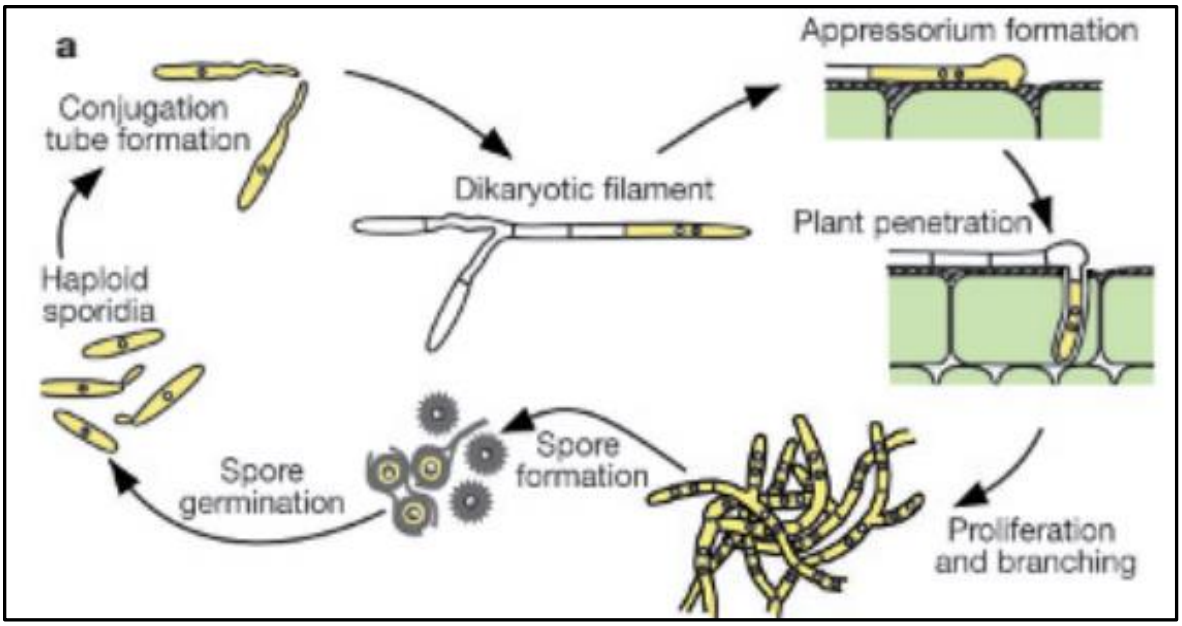

(Source: Kamper *et al*. 2006)

Figure 1.1. Disease cycle of corn smut caused by *Ustilago maydis* in maize

CHAPTER 2

COMPARATIVE GENOME ANALYSIS OF TEOSINTE AND B73 TO IDENTIFY CANDIDATE GENES WITHIN A 3.9 MBP REGION OF CHROMOSOME 9 CONFERRING RESISTANCE TO *USTILAGO MAYDIS*[1]

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ABSTRACT

Ustilago maydis, the causal agent of corn smut disease, is an important fungal pathogen infecting all aerial plant parts and induces local tumors. Significant corn yield losses caused by *U. maydis* and the lack of *U. maydis*-resistant maize cultivars necessitate identification of new sources of resistance to this pathogen. Phenotypic evaluation identified two near-isogenic lines (NILs) in a maize (B73) x teosinte (TIL11; *Zea mays* ssp. *parviglumis*) introgression population that are resistant to *U. maydis*. Maize B73 is the susceptible parent, while teosinte TIL11 is the resistant parent. Genotypic analysis of the two resistant NILs identified a 3.9 Mbp teosinte introgression on the short arm of chromosome 9 that was present in the two resistant NILs and absent in the susceptible NILs, suggesting the teosinte introgressed region from the resistant teosinte TIL11 parent is responsible for the resistant phenotype. Comparative analysis of TIL11, B73 and 25 nested association mapping population founder lines (B73- 25 NAM lines) identified 69 genes in the 3.9 Mbp introgression. Two of the genes were unique to TIL11, three genes were partially deleted in the B73-25 NAM lines, and sixty four genes were present in both TIL11 and B73-25 NAM lines. One of the genes unique to TIL11 (Peptidase_S10) was associated with plant defenses, and one of the genes partially deleted in B73-25 NAM lines (rice diacylglycerol kinase gene OsBIDK1) was associated with disease resistance. Twenty two genes identified in both TIL11 and B73-25 NAM lines were associated with disease resistance, pathogenesis related or defense response genes. Of the twenty two genes, four genes were classic resistance genes (R-genes), one was a pathogenesis related gene (PR proteins) and 17 genes were defense related genes. As a result, twenty-four candidate genes for resistance to *U. maydis* were identified in the TIL11 teosinte resistant parent. These findings offer insight into the evolutionary relationship

between disease resistance, pathogenesis related, and defense related genes in cultivated maize and a maize wild relative, *Zea mays* ssp. *parviglumis* and provide foundational knowledge of candidate genes underpinning resistance to *U. maydis*.

Keywords: *Ustilago maydis*, *Zea mays* ssp. *parviglumis*, Teosinte Inbred Line, Maize, Comparative analysis, Disease resistance genes, Pathogenesis related genes, Defense genes

INTRODUCTION

Maize, also known as corn, is a widely cultivated cereal crop used for food, feed, fodder, and biofuel (Cassidy et al. 2013). With global cultivation in tropical, subtropical, and temperate climates, the demand for maize is expected to increase due to its multifarious uses and the projected global population growth to 9 billion by 2050 (Rosegrant et al. 2009). To meet this demand, an annual corn production rate of 2.4% is needed, higher than the current rate of 1.6%. However, biotic and abiotic stresses remain a significant constraint in maize production systems (Rasmann et al. 2005; Davila-Flores et al. 2013). Modern maize inbred lines are also vulnerable to fungal and bacterial plant pathogens.

One of the major fungal diseases affecting maize yield is corn smut caused by *Ustilago maydis*. Despite various management practices such as seed treatment, sanitation, and crop rotation, this disease continues to be particularly destructive in developing countries. Modern maize has lost some of its natural defenses, leading to a narrow genetic base. Many resistance genes (R-genes), pathogenesis-related genes (PR proteins), and other defense-related genes that combat pathogens such as *U. maydis* have been lost or weakened during the domestication process (Chen et al. 2015). Additionally, population genetics comparison of domesticated maize and its wild ancestor, teosinte, has revealed evidence of selection in multiple genomic regions,

loss of genetic diversity during domestication, and post-domestication gene flow from teosinte into maize, which has enhanced maize's adaptation to diverse environments (Hufford et al. 2012). Since maize inbred lines are susceptible to *U. maydis* and corn smut causes significant economic losses, it is necessary to characterize other sources of resistance to *U. maydis*. Strengthening the genetic system is likely the most practical solution, and allelic diversity is essential to make the genetic system broad and versatile.

During natural selection, wild progenitors have evolved and retained many useful alleles as an integral part of their genomic constitution. Allelic diversity and adaptive genes are assumed to be more prevalent in wild progenitor teosintes, as they have been shown to thrive well under extreme conditions brought about by environmental and biological stresses. Disease resistance genes, such as classic resistance genes (R-genes), pathogenesis-related proteins, and other defense genes, have been introgressed from wild progenitors for rust, downy mildew, broomrape, powdery mildew, Verticillium wilt, head and stalk rot, and *Phomopis* (Chandler et al. 1986; Sujatha et al. 2008; Seiler 2010; Liu et al. 2010; Christov 2012; Nenova et al. 2014). Teosinte lines have been shown to provide resistance to several pathogens (Wei et al. 2001; Hajjar and Hodgkin 2007; Maxted and Kell 2009; Chavan and Smith 2014; Singh et al. 2021; Sahoo et al. 2021). Strategies to harness resistant traits for crop improvement show clear promise, as exemplified by the identification of gray leaf spot resistance quantitative trait loci (QTLs) using a population of near-isogenic lines derived from a cross between maize and TIL11 (*Z. mays* ssp. *parviglumis*) (Lennon et al. 2016). Similarly, a set of 169 backcross inbred lines derived from Maize x teosinte hybridization showed differential response to *Rhizoctonia* and enabled the identification of seven QTLs for banded leaf and sheath blight resistance when investigating BC_1F_5 lines derived from crossing maize and *Z. mays* ssp. *parviglumis* (Adhikari 2020).

In this study, TIL11 was used as a potential source of resistance to *U. maydis*. There are several subspecies of *Zea mays* identified as teosinte. Genetic analysis suggests that *Zea mays* ssp. *parviglumis* is the subspecies most closely related to domesticated maize (Doebley 1990; Matsuoka et al. 2002). A population of 900 NILs were developed from a cross between TIL11 and B73. NILs are genetic stocks that are genetically identical, but differ in one or more chromosomal segments. However, 900 NILs was a significant number to phenotypically characterize, so a minimum tilling path of 100 NILs was created. The 100 NILs had the entire teosinte genome, where each NIL carries a different region of the teosinte genome and associated markers. Phenotypic evaluation of the 100 NILs identified two maize-teosinte near-isogenic lines (NIL1 and NIL2) resistant to *U. maydis* (Chavan and Smith 2014), whereas the remaining 98 NILs were susceptible. NIL2 is a backcross four of NIL1. Genotypic analysis of the 100 NILs identified a 3.9 Mbp teosinte introgression region in the two resistant NILs on chromosome 9 of maize that was absent in the 98 susceptible NILs. This indicated that the resistance observed for the two NILs is a result of the teosinte introgressed region from the resistant teosinte TIL11 parent.

To identify and characterize candidate genes in the 3.9 Mbp teosinte region potentially responsible for the resistance phenotype of the two NILs, comparative analysis of the TIL11 and B73-25 NAM (Nested Association Mapping population) genomes was performed. The NAM population consists of twenty-five founder inbred lines (each NAM parental inbreds were crossed to B73) that were strategically selected from a larger association panel (Flint-Garcia et al. 2005) to represent the breadth of maize diversity (Yu et al. 2008).

In this study, 25 NAM lines were included in the comparative analysis as several studies in the past have demonstrated that a single genome is not representative of a species' diversity

(Bayer et al. 2020; Woodhouse et al. 2021). Using a broader pool of resources that consists of the B73 genome would make the process of identifying the genes unique to TIL11 more robust. Initially, the whole genome sequence of TIL11 was not available, but the whole genome sequence of TIL11 was made available by our collaborator Dr. Hufford at Iowa State University (Hufford et al. 2021). Comparative genomic analysis is useful for elucidating the function of genes, studying evolutionary history, and ordering and editing contigs (physical and sequence). Conserved regions between divergent genomes can be assessed by aligning genetic maps or comparing genomic sequences (Soderlund et al. 2006). The accurate detection of orthologous segments or syntenic regions plays an important role in comparative genomics (Hachiya et al. 2009). It is useful for inferring genome rearrangement scenarios and computing whole-genome alignments. As a first step towards a deeper evolutionary understanding of the genes in TIL11, an accurate identification of syntenic orthologs and comparisons of orthologous regions were performed using CoGe GEvo (comparative genomics genome evolution analysis tool) SynFind to elucidate the evolutionary fate of genes (Tang et al. 2015). TIL11 was used as a reference genome, and aligned with the B73-25 NAM populations to better understand the differences and similarities of genes in the 3.9 Mbp region between TIL11 and B73-25 NAM lines.

The primary goals in this study were to:

1. Identify variation between TIL11 and B73-25 NAM lines within the 3.9 Mbp region of chromosome 9.
2. Select candidate genes in the 3.9 Mbp region present only in TIL11 or present in TIL11 and B73-25 NAM lines using Pfam domains associated with disease resistance, pathogenesis related or defense genes.

To achieve these goals, a combination of computational and molecular techniques were used to identify and characterize the candidate resistance genes in the 3.9 Mbp teosinte introgressed region present in the two resistant NILs.

Hypothesis

Genes in the 3.9 Mbp region of chromosome 9 in TIL11 and B73 correlate with resistance to *U. maydis* based on gene presence-absence variation and Pfam domains associated with disease resistance, pathogenesis, and defense genes, facilitating the identification of candidate resistance genes in the introgressed region.

MATERIALS AND METHODS

Comparative Genome Analysis of TIL11 and B73-25 NAM Founder Lines

The TIL11 (PI 384071) sequence was employed as the reference genome for comparative analysis of TIL11 and B73-25 NAM founder lines. Dr. Hufford at Iowa State University conducted PacBio sequencing of TIL11 and made the sequence accessible to us. The 25 NAM genomes were sequenced to high depth (63-85X) using PacBio long-read technology, assembled into contigs using a hybrid approach, and ordered into pseudomolecules using linkage data from the 25 NAM recombinant inbred lines and maize pan-genome anchor markers (Hufford et al. 2021). The B73-25 NAM founder line sequences were obtained from the MaizeGDB (https://www.maizegdb.org/). B73 version 5 was utilized to determine the genomic coordinates of TIL11 in the 3.9 Mbp target region of chromosome 9. The chromosome coordinates were identified by using the SynFind tool to flank the B73 chromosome coordinates.

TIL11 Candidate-gene Discovery Using Syntenic Dotplots

The utilization of SynMap, an online comparative genomics tool (CoGe), enabled the generation of whole genome syntenic dotplots (Lyons and Freeling 2008). To compare overall genomic synteny between TIL11 and B73- 25 NAM founder lines in order to identify candidate genes for resistance to *U. maydis*, dotplots were created by comparing the two genomes. It is worth noting that only syntenic gene pairs identified through collinearity were represented on the dotplot (Tang and Lyons 2012).

TIL11 Candidate-gene Discovery Using Syntenic Orthologs

To identify syntenic orthologs between a preliminary gene model annotation of TIL11 and gene model annotations of domesticated maize genomes (B73 version 5 and 25 NAM founder lines) (Hufford et al. 2021), the CoGe SynFind tool (Lyons and Freeling 2008) was employed, utilizing TIL11 as the query genome. The TIL11 genomic coordinates for the 3.9 Mbp target region on chromosome 9, corresponding to the known coordinates in B73 version 5 were determined by extracting syntenic gene models between B73 and TIL11 flanking this region in B73 from the SynFind analysis (Tang 2010) and determining the TIL11 coordinates within the flanking genes.

BLAST and Visual Curation Using Comparative Genomics Genome Evolution Tool

To identify TiL11 loci missing syntenic orthologs in the 3.9 Mbp target region in B73 (domesticated maize), BLAST and visual curation were performed using CoGe GEvo (Schnable and Lyons 2011) (https://genomevolution.org/coge/GEvo.pl). Indels in proximity to TiL11 candidate genes were determined through visual inspection with CoGe GEvo. The predicted transcript sequences of TIL11 genes on chromosome 9 were converted to protein sequence with

Emboss transeq (http://emboss.sourceforge.net/apps/cvs/emboss/apps/transeq.html). Pfam (protein family) domains in the TIL11 protein sequences were obtained by aligning Pfam motifs (https://pfam.xfam.org/ doi: 10.1093/nar/gkaa913) using hmmer3 (http://hmmer.org) with the -E parameter set to ≤ 1e-5.

Ranking of Identified Candidate Genes Associated with Resistance to *Ustilago maydis*

Candidate genes identified as a result of comparative analysis of TIL11 and B73-25 NAM founder lines were ranked according to the following criteria: 1) presence-absence variation of domesticated maize syntenic orthologs in the 3.9 Mbp target region in TIL11, 2) presence-absence variation of indels in B73-25 NAM lines or in proximity to the TIL11 3.9 Mbp target region, and 3) presence of Pfam domains associated with disease resistance. The identified genes encoding Pfam domains with functions related to disease resistance, pathogenesis-related genes, and defense response genes corresponding to their proteins were researched in the literature. The genes unique to TIL11 and partially deleted in the B73-25 NAM lines were classified as first-priority candidate genes. The candidate genes identified in both TIL11 and B73-25 NAM lines were classified as second-priority candidate genes. Several candidate genes were identified in the second-priority group. As a result, these genes were further divided into three priority groups based on function and included classic disease resistance genes (priority group 1), pathogenesis-related genes (priority group 2), and defense-related genes (priority group 3).

RESULTS

Syntenic Dotplot Between TIL11 and B73-NAM Genomes

The syntenic dotplot (Figure not shown**)** provided a graphical representation of the comparison of maize chromosome 9 (B73-25 NAM lines) and *Zea mays* ssp. *parvigumis* (TIL11). The dotplot allowed a convenient and efficient method to visualize the genome-wide synteny between two genomes and to identify regions of variations between them. The dotplot depicts the regions of similarity between the two genomes, as identified through collinearity, represented by the green line. However, it is also important to note that the discontinuities in this syntenic line represent regions of genomic variations at a given locus between the two genomes. These variations can be the result of either deletions or translocations. The red highlighted box is the 3.9 Mbp teosinte introgressed region. As evident from the dotplot, the syntenic regions encompass the entire chromosome 9, highlighting the similarity in the overall structure of the TIL11 and B73-25 NAM genomes. However, there are some deletions (gaps between the green lines) between TIL11 and B73-25 NAM_refgen, and a few chromosome pieces have undergone changes in location. The red highlighted area in the dotplot, represents the 3.9 Mbp region in B73-version 3, which corresponds to a 1.5 Mbp region in B73-version 5 and TIL11 on chromosome 9. Despite the presence of deletions and changes in location, no insertions, deletions or translocations were observed.

Identification of Genomic Coordinates of 3.9 Mbp Introgression Region in TIL11

The genomic coordinates of the 3.9 Mb introgressed region on chromosome 9 from TIL11 were identified by comparing the TIL11 and B73 genomes using bioinformatics tools and techniques including whole genome syntenic dotplots, generating syntenic orthologs and the

SynFind tool. The coordinates for TIL11 were syntenic to the B73 target region of chromosome 9. As a result, the 3.9 Mbp region was identified in TIL11 and was analyzed to identify candidate genes associated with resistance to *U. maydis*.

Identification of Unique Candidate Genes in TIL11 with Putative Disease Resistance Function

Comprehensive analysis of the 3.9 Mbp region of TIL11 and B73-25 NAM lines led to the identification of 69 genes in the region. Five of the 69 genes were unique to TIL11 or partially deleted in B73-25 NAM lines (Table not shown). The remaining 64 genes were identified in both TIL11 and B73-25 NAM lines. Further examination of these unique genes revealed that two genes were missing syntenic orthologs in B73-NAM lines and encode RNase_PH_C and Pfam domain of Peptidase_S10 (**Figure 2.1**). One gene with a Pfam domain was associated with plant defense related genes.

In addition, gene encoding Pfam domain Nodulin-like was partially deleted in all NAM/B73 lines. A gene that was partially deleted in 13 B73- NAM lines encoded Pfam domain DAGK_cat (Diacylglycerol kinase catalytic domain). Gene encoding the GMC_oxred_C and GMC_oxred_N Pfam domain was partially deleted in 10 lines including B73. Gene with Pfam domain Diacylglycerol kinase catalytic domain was associated plant with defense genes.

Identification of Candidate Genes in both TIL11 and B73-NAM Lines Associated with Disease Resistance Function

Of the 64 genes identified in both TIL11 and B73-NAM lines, 22 genes encoded Pfam domains known to be associated with disease resistance **(Table 2.1)**. In order to better understand the role of these genes in disease resistance, the genes were further classified into

three distinct categories, priority group 1, priority group 2, and priority group 3. This classification was based on the Pfam domains encoded by the genes and their associated putative function in plant defenses. Four genes were categorized as priority group 1, and encode Pfam domains associated with classic resistance genes (R-genes) such as serine-threonine protein kinase (PK_Tyr_Ser-Thr), protein kinase (Pkinase), Kinase-like, and Enhanced Disease Resistance 1 (EDR1) protein kinase. These R-genes are known to respond to the products of avirulence genes expressed by pathogens during infection and initiate signal transduction to activate defenses (Friedman and Baker 2007; Yahiaoui et al. 2009). One candidate gene was grouped into priority group 2, encoded a Pfam domain associated with pathogenesis related proteins (PRs), which are a class of proteins that accumulate in response to biotic and abiotic stresses to protect plants from damage (Li et al. 2021). Seventeen candidate genes were grouped into priority group 3, as they encoded Pfam domains associated with plant defense or immunity (**Table 2.1**). These data confirmed that some of the disease resistance, defense and pathogenesis-related genes are conserved in both TIL11 and B73-25 NAM lines.

DISCUSSION

This study presents a comparative analysis of the 3.9 Mbp genomic region on chromosome 9 identified in TIL11 and B73-25 NAM founder lines. Comparative analysis of the resistant (TIL11) and susceptible (B73-NAM) parental lines facilitated the identification of candidate genes in the 3.9 Mbp region associated with resistance to *U. maydis* in the two resistant NILs. To the best of our knowledge, this is the first study that aims to identify and prioritize functional candidate genes from TIL11, a maize wild ancestor, that are potentially associated with plant resistance and defenses to *U. maydis*.

SynFind, a web-based tool, was utilized to determine gene presence-absence variation in domesticated maize B73-25 NAM lines, and TIL11 (Tettelin et al. 2005). By utilizing the TIL11 genome as the query, presence-absence variation of orthologous sequence or genes were detected to identify genes unique to TIL11 and absent in B73-25 NAM lines, and genes conserved in both TIL11 and B73-25 NAM lines.

Two genes in the 3.9 Mpb region were unique to TIL11. These genes encoded pfam domains associated to disease resistance. This suggests that the domestication and selection process of maize has led to the loss of certain genes in B73-25 NAM lines, as previously reported by Chen et al. (2020). Identification of a peptidase gene aligns with previous research on the role of peptidases in disease resistance. Liu et al. (2008) demonstrated that the overexpression of OsBISCPL1, a rice peptidase S10/subtilisin-like protease gene, in *Arabidopsis* plants enhanced disease resistance against *Pseudomonas syringae* and *Alternaria brassicicola*, accompanied by the induction of defense-related genes. Similarly, the overexpression of CDR1, an *Arabidopsis* aspartic peptidase gene, enhanced resistance to a virulent *P. syringae* isolate (Xia et al. 2004). Additionally, Pearce et al. (2010) discovered that GmSubPep, a 12-aa peptide derived from a member of the subtilisin-like protease family in soybean, induced the expression of defense-related genes. Peptidase genes, such as Peptidase_S10, that are downregulated in W28oX rice plants play an important role in basal defense response against both rice fungal and bacterial pathogens (Chujo et al. 2013). Furthermore, oligopeptidases are involved in quantitative disease resistance against *Sclerotinia sclerotiorum*, a fungal pathogen that causes white mold and watery soft rot in vegetables (Badet et al. 2017). These findings suggest that peptidase genes identified in the TIl11 3.9 Mpb region may play a role in plant defense and may serve as a good candidate genes for resistance to *U. maydis.*

Further, three genes were partially deleted in 13 B73/NAM lines susceptible to *U. maydis*. One of the three genes encoded a diacylglycerol kinase catalytic Pfam domain. The diacylglycerol kinase (DAGK) pathway is a critical component of the lipid signaling pathway and plays a vital role in various aspects of plant growth and development, such as hormone response, guard cell closure, and defense responses against biotic and abiotic stresses (Laxalt and Munnik 2002; Meijer and Munnik 2003; Munnik 2001; Testerink and Munnik 2005). Activation of the DAGK signaling pathway has also been linked to the occurrence of oxidative burst, the induction of mitogen-activated protein kinase cascades, and the activation of disease resistance responses in plant cells upon exposure to pathogen-derived avirulence factors (Andersson et al. 2006; de Jong et al. 2004; Joosten and de Wit 1999). Furthermore, protein kinase C activated by DAGK is involved in the regulation of defense responses induced by elicitors (Subramaniam et al. 1997). Zhang et al. (2008) demonstrated that the overexpression of a rice diacylglycerol kinase gene OsBIDK1 enhance disease resistance in transgenic tobacco. This suggests that the DAGK signaling pathway plays an important role in defense responses and may be a potential target for improving plant disease resistance. Partial deletion of the DAGK catalytic domain in 13 B73-NAM lines highlights the potential impact of the deletion on disease resistance in these susceptible lines and calls for further studies to understand the mechanisms involved. These findings suggest that DAGK may be a good candidate gene for resistance to *U. maydis* particularly due to the presence-absence variation of this gene in TIL11 and B73-25 NAM lines.

Comparative analysis identified 22 candidate genes in the 3.9 Mbp region in both TIL11 and B73-25 NAM lines. These genes included R-genes, PR-genes, and defense genes with functions related to plant growth, plant development, and defense responses against biotic and abiotic stresses. Notably, while resistance for both NILs likely originated from the TIL11

resistant parent, some of the candidate genes were present in both TIL11 and B73-25 NAM lines. One potential explanation for this could be the presence of suppressors of resistance genes in the susceptible B73 genotype that may inhibit the expression of resistance genes against *U. maydis* in the susceptible B73 parent. The absence or mutation of certain genes in susceptible B73 parent could potentially cause this suppression. Prior research identified suppressors of stem rust resistance genes 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). Additionally, these conserved genes may have lost their function during the process of domestication and evolution of the B73 genome.

Twenty-four underlying candidate genes potentially associated with resistant to *U. maydis* were identified through comparative genome analysis of the 3.9 Mbp genomic region of TIL11 and B73-25 NAM lines. The primary objective of this study was to identify the potential candidate genes that are unique to TIL11 or present only in TIL11, which indicates that these genes may contribute to the resistance in maize-teosinte NILs, as B73 is known to be susceptible to *U. maydis*. However, the results of this study also revealed that some of the potential candidate genes are partially deleted in B73-25 NAM lines, and some of the genes are present in both TIL11 and B73. Thus, it is challenging to draw conclusions regarding the potential candidate genes involved in resistance to *U. maydis* based on this study alone.

The genome sequence of TIL11 was invaluable in identifying presence-absence variation in TIL11 and B73-25 NAM lines. Nevertheless, this research does not demonstrate that the 24 candidate genes are responsible for the resistance phenotype in the TIL11 and the two resistant NILs. It is unclear whether the candidate genes that are unique to TIL11, partially deleted in B73-25 NAM lines, or present in both TIL11 and B73-25 NAM lines are expressed. R- gene

proteins, defense related proteins, and PR proteins are functionally diverse, inducible during pathogen attack and regulated by signaling compounds. Therefore, to select candidates from the 24 genes present in both TIL11 and B73-25 NAM lines, differential expression (RNA-sequencing) of these common genes after infection with *U. maydis* will provide insight into their function and potential role in disease resistance. We hypothesize that the log-fold change value of candidate genes unique to TIL11, partially deleted in B73-25 NAM lines or present in both TIL11 and B73-25 NAM lines will be significantly higher in TIL11 and NILs as compared to B73. This highlights the importance of confirming the functions of these candidate genes in order to improve resistance to *U. maydis* (Laxalt and Munnik 2002; Meijer and Munnik 2003; Munnik 2001; Testerink and Munnik 2005; Andersson et al. 2006; de Jong et al. 2004; Joosten and de Wit 1999; Subramaniam et al. 1997; Zhang et al. 2008).

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Table 2.1. Genes present in both TIL11 and B73-25 NAM lines encoding Pfam domains associated with disease resistance, pathogenesis related or plant defense function in the 3.9 Mbp region of chromosome 9

Pfam domain(s)	Functions related to disease resistance, pathogenesis related or plant defense	References
Priority group 1: Classic resistance genes		
1. PK_Tyr_Ser-Thr	Protein tyrosine and serine/threonine kinases play a crucial role in recognizing pathogenic ligands on the cell surface and the subsequent activation of intracellular defense mechanisms. In plants, the receptor-like cytoplasmic kinase (RLCK) known as Botrytis-induced kinase 1 (BIK1) has been found to possess dual-specificity kinase activity, with both tyrosine and serine/threonine kinase activities being essential for its function in innate immunity in Arabidopsis.	Lin et al. 2014
2. Pkinase	Protein kinase genes play a crucial role in the disease resistance pathway and are essential for the proper functioning of R genes in plants.	Dangl and Jones 2001
3. Kinase-like	The Xa21 gene in rice, which confers resistance to the pathogen *Xanthomonas oryzae* pv. *oryzae* race 6, has been found to encode a kinase-like protein. This protein plays a crucial role in the disease resistance pathway and is essential for the function of plant resistance genes.	Song et al. 1995
4. EDR1	The expression of plant defensins in response to invasion by the fungal pathogen *Colletotrichum tropicale* depends on the EDR1 (Enhanced Disease Resistance 1) protein kinase and the ORA59 transcription factor in *Arabidopsis thaliana*.	Kosaka et al. 2020
Priority group 2: Pathogenesis related proteins		
1. Per1	The Per1 (peroxidredoxin antioxidant) gene plays a crucial role in augmenting tolerance to kernel stress and resistance against aflatoxin in plants.	Chen et al. 2007
Priority group 3: Other genes related to defense		
1. Pentatricopeptide repeat (PPR); PPR_1; PPR_2; PPR_3; PPR_long	The semi-quantitative RT-PCR technique was utilized to assess the transcript levels of pentatricopeptide repeat-containing (PPR) proteins in response to *Ralstonia solanacearum* (Rs) infection. The results revealed an upregulation in PPR transcript levels, indicating that this protein plays a role in the stress tolerance of potato plants in response to Rs infection.	Park et al. 2016
2. DYW_deaminase	Gene encoding for DYW_deaminase domain-containing protein was shown to reduce pathogen infection in wheat.	Hayes et al. 2013, 2020
3. Homeodomain	Certain homeodomain proteins play a crucial role in regulating the transcription of defense-related genes and controlling programmed cell death in response to various stressors.	Korfhage et al. 1994; Mayda et al. 1999
4. Homeobox_KN	Homeobox_KN proteins have been demonstrated to contribute to the resistance of cotton to fungal pathogens such as *Verticillium dahliae* and *Botrytis cinerea.*	Gao et al. 2016
5. WD40	The presence of WD40 repeat (WDR) containing proteins has been linked to a variety of fundamental mechanisms, including signal transduction, chromatin modification, and	Nocker and Ludwig 2003;

	transcriptional regulation. These proteins have also been found to play a role in a wide range of plant processes, including innate immunity.	Perfus- Barbeoch et al. 2004
6. eIF2A	The eukaryotic initiation factor 2 (eIF2) has been the subject of numerous reviews, focusing on its role in plant R and/or recessive resistance genes. It has been demonstrated that mutations in the genes for eIF2B can lead to significant human health conditions.	Robaglia and Caranta 2006; Wang and Krishnaswamy 2012
7. Remorin_C	The protein known as remorin is a plant-specific protein that is located on the plasma membrane and has been linked to quantitative disease resistance in maize. This specific gene has been identified as playing a role in this process.	Jamann et al. 2016
8. THOC7	Down-regulation of the THO complex subunit 7 (THOC7) gene was likely involved in maize defense responses.	Asters et al. 2014
9. Histone	The protein histone plays a role in a variety of plant defense mechanisms.	Palma et al. 2010; Xia et al. 2013
10. NAC	The NAC (NAM, ATAF and CUC) family is one of the largest groups of plant-specific transcription factors. These proteins play a crucial role in regulating plant immunity by influencing the signaling of hormones, which are essential for triggering plant immune responses.	Yuan et al. 2019; Lee et al. 2017
11. Inhibitor_I9	The protease inhibitors, or PIs, found in plants are known to play an important role in defense mechanisms against pathogens, pests and abiotic stresses. These proteins work by inhibiting the activity of proteases, which are enzymes that break down proteins, thereby protecting the plant from these harmful agents.	Fan et al. 2019
12. PA	Several proteases, enzymes that break down proteins, have been identified to play a role in activating plant defense mechanisms. These proteases have been found to be involved in the plant's response to pathogens and pests, as well as to abiotic stressors.	Xia 2004
13. Peptidase_S8	The mature form of the S8 protein has been found to possess proteolytic activity and has been shown to trigger defense responses in plants.	Chalfoun et al. 2013; Figueiredo et al. 2018
14. TPR_1; TPR_2; TPR_19	The Tetratrico Peptide Repeats (TPRs) proteins have been found to play a role in both hormone signaling and defense responses in plants.	Takahashi et al. 2003
15. AMP-binding;AMP-binding_C	OsBIABP1, an antimicrobial peptide binding protein, may play a role in defense response regulation through the salicylic acid and/or jasmonic acid/ethylene signaling pathways.	Zhang et al. 2009
16. YT521-B homology (YTH)	The research has established that the YTH gene is a crucial factor in the plant's ability to withstand both biotic and abiotic stressors. YTH domain proteins have been found to be essential for rice growth and stress response.	Ma et al. 2022
17. Amino_oxidase	Several studies have demonstrated that in plants, copper amine oxidases (CuAO) and polyamine oxidases (PAO) play a crucial role in both preformed and inducible defense	Cona et al. 2006

	responses that take place in the apoplast following biotic stress. This is primarily achieved through the production of hydrogen peroxide (H_2O_2).	
18. NAD_binding_8	The molecule NAD (Nicotinamide Adenine Dinucleotide) plays a role in activating defense responses in *Arabidopsis thaliana*. It has shown that upon pathogen infection, NAD is released into the extracellular space in sufficient concentrations to trigger defensive mechanisms.	Alferez et al. 2018
19. IQ	A novel calmodulin-binding nuclear protein known as IQD1 in Arabidopsis plays a role in stimulating the accumulation of glucosinolates and activating plant defense mechanisms.	Levy et al. 2005

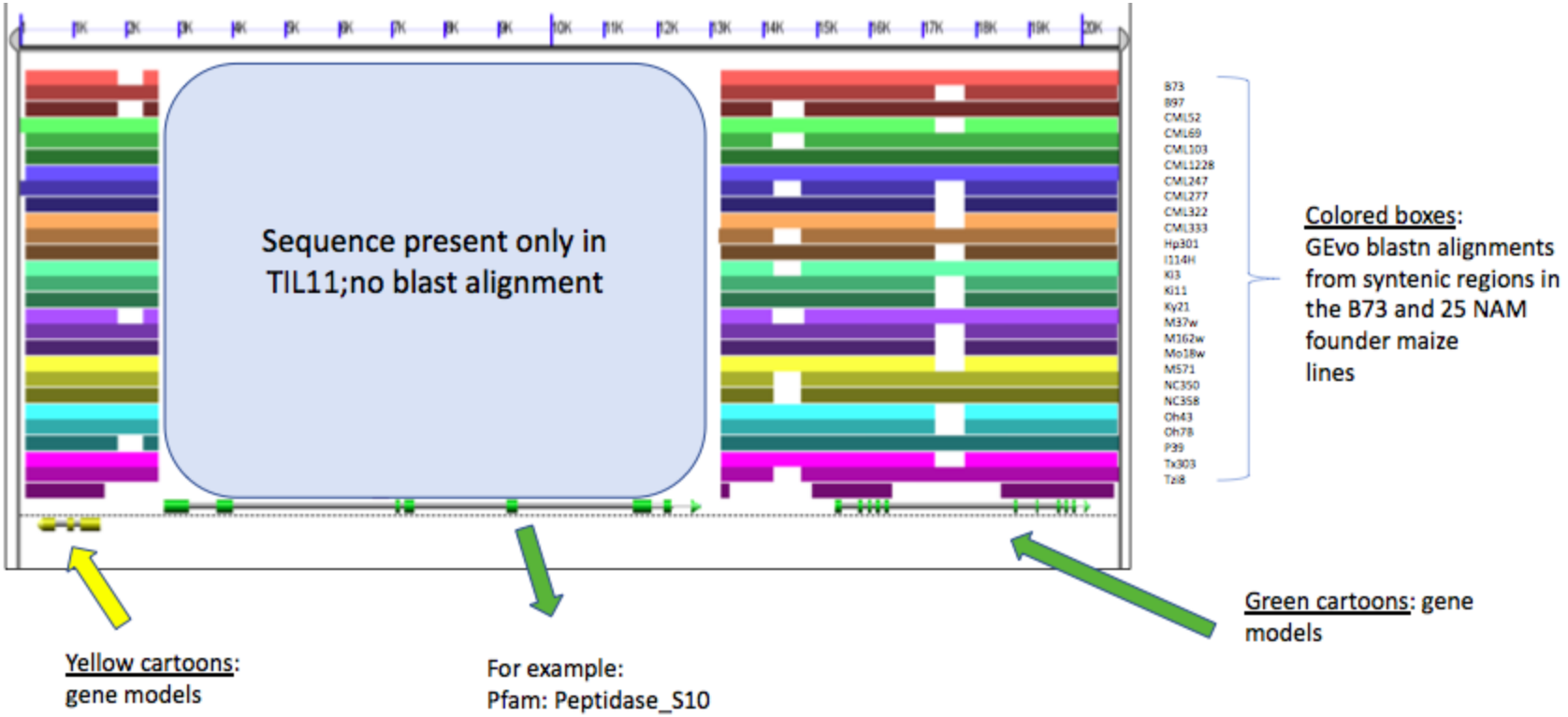

Figure 2.1. Gene model of the 3.9-Mbp teosinte introgressed region in chromosome 9 of TIL11 reference genome aligned with B73 and 25 NAM lines. Different colors represent each of the founder lines. The arrows in green and yellow depicts gene models and exons. This figure represents TIL11 sequence that lacks syntenic orthologs across B73 and 25 NAM lines and TIl11 sequence that has syntenic orthologs across B73- 25 NAM lines. An example of a gene that was unique to TIL11 and associated with disease resistance function (Peptidase S10) is shown at the bottom of the light blue box.

CHAPTER 3

TRANSCRIPTOME ANALYSIS TO IDENTIFY DIFFERENTIALLY EXPRESSED GENES ASSOCIATED WITH RESISTANCE TO *USTILAGO MAYDIS* IN TEOSINTE AND TWO MAIZE-TEOSINTE NEAR-ISOGENIC LINES[2]

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ABSTRACT

Corn smut, a fungal disease caused by the biotrophic pathogen *Ustilago maydis* affects corn production worldwide. Since *U. maydis* causes huge corn yield losses on modern maize lines that are susceptible to this pathogen, there is a need to identify new sources of resistance. In previous work, a cross between a wild maize ancestor, *Zea mays* ssp. *parviglumis* and the domesticated maize inbred B73 produced 100 near-isogenic lines (NILs). From the genotypic and phenotypic evaluation, two NILs (NIL1 and NIL2) had a 3.9-Mbp teosinte introgressed region, contributing to the resistance in two NILs. Further, comparative genome analysis of a potentially resistant TIL11 and the susceptible line- B73 in the 3.9 Mbp region identified 24 candidate genes that were either unique to TIL11, partially deleted in some of the B73- 25 NAM lines or present in both TIL11 and B73-25 NAM lines in the 3.9 Mbp region of chromosome 9. To determine which candidate genes were expressed and potentially contributing to the resistance phenotype, RNA-sequencing was performed for TIL11, B73 and the two NILs. Seedlings of TIL11, B73, and two NILs were inoculated with 200 µl of *U. maydis*. Leaf samples from three biological replicates were collected from each genotype 12 hours post-inoculation. A similar experiment was conducted for mock inoculation. Total RNA was extracted and sequenced using a Nova seq 6000 platform. Twenty-four genes were differentially expressed in B73, TIL11, and two NILs in response to *U. maydis*. Four of the 24 genes were upregulated and significantly expressed in TIL11 and two NILs. Three of the upregulated genes encode protein family (Pfam) domains associated with disease resistance (1. PK_Tyr_Ser-Thr/Pkinase; 2. LTP_2/Tryp_alpha_amyl ; 3. TPR_1). However, fourth gene (No Pfam) did not encode a Pfam domain. Expression of the four genes was validated with qRT-PCR. The qRT-PCR expression results were consistent with the RNA-seq expression patterns for all four genes. Therefore, these four genes were identified as

promising candidates in the 3.9 Mbp region potentially associated with disease resistance to *U. maydis*. As a result, four genes were selected for functional characterization to validate their function in resistance to *U. maydis*.

Keywords: *Ustilago maydis, Zea mays* ssp. *parviglumis,* Near-isogenic lines*,* RNA-sequencing qRT-PCR, Protein family

INTRODUCTION

Ustilago maydis causes corn smut disease on all types of maize organs resulting in substantial yield loss and reduced quality (Ruan et al. 2021; Dean et al. 2012). However, maize ears, tassels, leaves and stalks at the seedling stage are more susceptible to corn smut. *U. maydis* has a biphasic life cycle consisting of a non-pathogenic phase of yeast-like growth of haploid cells, called sporidia and a pathogenic phase. The pathogenic phase begins with the formation of a dikaryotic filament by fusion of mating filaments from compatible haploid sporidia (Kahmann et al. 1995). The infection starts with development of an appressorium, a specialized infectious structure that penetrates the epidermal cells (Snetselaar and Mims 1993; Lanver et al. 2014). The infectious hyphae creates a biotrophic interaction zone through the invagination of the plant plasma membrane, which serves as the main interaction site for suppressing plant defense and acquiring nutrients.

Once pathogen acquire the capacity to suppress primary defenses, plants develop more specialized mechanisms to defend themselves from disease (McDowell et al. 2003). The first step in the activation of plant defense response is triggered by recognition (directly or indirectly) of the pathogen's avirulence (Avr) gene by the plant's resistance gene (R-gene). This recognition results in the activation of plant defenses and is qualitatively controlled (Dangl and Jones 2001;

Cui et al. 2015). For biotrophs, R-gene-mediated resistance results in resistance by restricting fungal growth in infected cells that have undergone hypersensitive cell death (Chisholm et al. 2006). During the early phase of maize infection, *U. maydis* is recognized by the plant via conserved molecular patterns (PAMPs). This leads to salicylate-dependent defense responses, a typical response of plants to biotrophs (Doehlemann et al. 2008; Glazebrook 2005). It has been shown that 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).

In contrast to several other biotrophic interactions, the maize-*U. maydis* pathosystem lacks gene-for-gene interactions as it relates to R-gene-Avr-gene interactions. Cultivated maize resistance to *U. maydis* is a polygenic, quantitative characteristic (Schurack et al. 2021). Quantitative Disease Resistance (QDR) is characterized by many genes with small to moderate effects leading to a continuous distribution of susceptible to resistant phenotypes (Niks et al. 2015; Poland et al. 2009; Roux et al. 2014). In addition, QDR genes confer broad-spectrum resistance and have been associated with durable resistance in many cases (Nelson et al. 2018). Although *U. maydis* is considered the predominant model organism of biotrophic plant pathogens, resistance to *U. maydis* has been rarely described (Baumgarten et al. 2007; Lubberstedt et al. 1998). Baumgarten et al. (2007) and Brefort et al. (2009) reported a few QDR loci conferring resistance to *U. maydis* that contain genes with a known role in defense against pathogens, such as nucleotide-binding domains, leucine-rich repeat containing Nucleotide oligomerization domain-like receptors (NLRs), a pathogenesis-related protein, a chitinase, a basal antifungal protein and a wound-inducible protein. In rare cases, NLR genes can also underlie QDR (Barbacci et al. 2020; Poland et al. 2009), which has led to the hypothesis that

allelic variants, *i.e.,* weak alleles of R-genes, can cause incomplete resistance. The molecular functions underlying QDR seem to be highly diverse compared to qualitative resistance. In addition, as other responses to pathogen attacks, QDR may require the coordinated and early expression of plant defense mechanisms, including various defense-associated metabolites, proteins, or genes involved in other plant immunity pathways (Delplace et al. 2020). However, QDR remains poorly understood as the underlying complex genetic architecture has limited the molecular characterization of mechanisms involved, despite it determining the outcome of the majority of plant-pathogen interactions in crop plants (Bartoli and Roux 2017; Corwin and Kliebenstein 2017; Poland et al. 2009, and Roux et al. 2014).

One of the most effective and practical management methods is host resistance. The use of wild relatives as a source of resistance to different pathogens has proven to be very promising (Hajjar and Hodgkin 2007). Previous work identified two maize-teosinte near-isogenic lines (NILs) from a maize (B73)-teosinte (*Zea mays* ssp. *parviglumis*/teosinte inbred line- TIL11) population that were resistant to *U. maydis* (Chavan and Smith 2014). Genotypic analysis identified a 3.9 Mbp teosinte introgressed region in the two resistant NILs indicating that this region contributes to the resistance phenotype. Comparative genome analysis of TIL11 and B73 was performed to identify genes in the 3.9-Mbp region contributing to the resistance phenotype. This led to the identification of 24 candidate genes in the 3.9-Mbp region that are possibly contributing to the resistance observed for the resistant parent (TIL11) and the two resistant NILs (NIL1 and NIL2). Identification of candidate genes does not mean that these genes are actually expressed. Therefore, identification of candidate genes in the 3.9 Mbp teosinte introgression, coupled with RNA sequencing of the resistant parent (TIL11), susceptible parent (B73) and two resistant NILs will shorten the list of candidate genes that are associated with the resistance

phenotype. Comprehensive transcriptome analyses of the plant and the pathogen after pathogen inoculation is commonly used to provide new insights into the molecular mechanisms of plant resistance (Han et al. 2015). Analysis of the differentially expressed genes in resistant and susceptible cultivars has led 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).

In order to understand the molecular basis of resistance to *U. maydis* and to identify key functional genes from the 24 genes identified in the 3.9 Mbp introgression, we performed RNA-sequencing to identify differences in gene expression in TIL11, B73 and the two NILs.

The objectives of the study were to: 1) identify candidate genes either uniquely expressed in TIL11 and two NILs or expressed in all four genotypes (TIL11, B73 and two NILs) at 12 hrs post inoculation, 2) select candidate genes that are uniquely expressed in TIL11 or are significantly upregulated in TIL11 and the NILs in response to *U. maydis,* and 3) validate the selected candidate genes differential expression via qRT-PCR.

Hypothesis

Genes in the 3.9 Mbp introgression will be differentially expressed in TIL11, B73 and the two NILs after inoculation with *U. maydis*. Genes associated with resistance to *U. maydis* will be upregulated in TIL11 and the two NILs resulting in identification of candidate genes for resistance to *U. maydis*.

MATERIALS AND METHODS

Plant Materials and *Ustilago maydis* Inoculation

Four genotypes, including a maize inbred line (B73), TIL11, and two NILs (NIL1 and NIL2), were grown in a growth chamber for *U. maydis* inoculation. The plants were exposed to a

16/8 hour photoperiod and a day and night temperature of 28/22°C. Each experimental unit consisted of a single seedling, with 24 seedlings (12 control: 12 treatment) grown for each of the four genotypes. Two treatment groups were established: a) 12 seedlings inoculated with *U. maydis* strains, and b) 12 seedlings inoculated with water (mock-inoculated). Three biological replicates from each genotype were used for treatment and control groups.

A wild type *U. maydis* strain ½ (mating type a1b1) and a near isogenic strain 2/9 (mating type a2b2) were used for inoculation (Allen et al. 2011). Three potato dextrose agar plates were streaked with each of the *U. maydis* strains (a1b1 and a2b2). Single spores were cultured separately in three test tubes for each strain. Both strains were grown in potato dextrose broth at 30°C to an OD_{600} of 1.0. Cells were suspended in water to a final concentration of 1×10^6 cells/ml. The culm of the plants, just above the soil line, were injected with ~200 µl of cell suspension culture with a hypodermic needle 7-8 days after planting. Mock inoculations were also performed with ~200 µl of sterile water. Samples of mock-inoculated and treated maize seedlings were collected. In addition, phenotypic evaluation of these genotypes was performed each day for 21 days post inoculation using a disease rating scale of 0-5.

RNA Extraction, Library Preparation, and RNA Sequencing

Total RNA was isolated from three seedlings (~70 mg) obtained from B73, TIL11, and the two NILs 12 hours post inoculation for the control and treatment groups. Each leaf was placed in a separate 1.5 ml microcentrifuge tube, immediately frozen in liquid nitrogen, and stored at -80 °C. The leaves were homogenized in a microcentrifuge tube using a pestle and suspended in 1 ml of TRIzol reagent (Thermo Fisher Scientific, Waltham, USA). RNA was extracted for each sample as described by the manufacturer (Thermo Fisher Scientific, Waltham, USA). RNA was then resuspended in 30 µl of RNAse free water. The quality of the RNA was

first assessed with agarose electrophoresis. RNA integrity was also assessed by determining the RNA Integrity Number (RIN) value for each sample using the Agilent Bioanalyzer RNA Nano 6000 Assay Kit with the Agilent Bioanalyzer 2100 system (Agilent Technologies, Santa Clara, CA, USA) and then quantified with Qubit 4 Fluorometer (Invitrogen, United States). Total RNA was then sent to Iowa State University DNA sequencing Facility for library preparation and RNA sequencing.

Sequencing libraries were generated using a NEBNext Ultra II Directional RNA Library Prep Kit for Illumina (NEB, USA) according to the manufacturer's recommendations. For library construction, 15 µl of total RNA per sample was used, and messenger RNA (mRNA) was enriched from total RNA using oligo (dT) magnetic beads. Since rRNA makes up an overwhelming fraction of the total RNA, the mRNA should be enriched. Oligo (dT) beads were used for Stranded total RNA to accomplish mRNA enrichment. The first strand of cDNA was synthesized using NEBNext strand specificity reagent and NEBNext first strand synthesis enzyme mix. The second strand cDNA was synthesized using NEBNext second strand synthesis reaction buffer with dUTP Mix and NEBNext second Strand Synthesis Enzyme mix. The double-stranded cDNA was purified with NEBNext sample purification beads. After cDNA purification, the poly (A) tails were added and ligated to the sequencing adapter. The PCR amplification was purified using NEBNext sample purification beads. The quality of the library was then assessed on an Agilent Bioanalyzer DNA Chip. After the quality check, the libraries were pooled and then sequenced on a Nova-seq 6000 platform at the DNA facility at Iowa State University. Stranded total RNA sequencing with an rRNA facility depletion was performed on a NovaSeq 6000 SP flow cell with a 150-cycle paired-end read length (total cycles- 300).

Sequence Alignment and Bioinformatics Analysis

The original data contained sequencing adapters or low-quality reads. In order to ensure the quality and reliability of the analysis, the original data were filtered, which mainly involved removing reads with adapters, as well as reads with N (N indicates that the base information cannot be determined) and low-quality reads (qphred ≤ 20). Q20, Q30, and the GC content were calculated for the clean data. Reads were filtered across all 16 samples for each gene. All subsequent analyses were conducted with the clean data set. The fastq files were first remapped to an rRNA/tRNA fasta file, and then the reads were mapped to TIL11 reference genome to obtain positioning information for the reads in the reference genome using STAR (Dobin et al. 2013). The mismatch stringency was made somewhat lenient to a mismatch value of 5. Mapped library bam files were then run through the program subread Feature Counts (Liao et al. 2014) to calculate the fragments per kilobase of transcripts per million mapped reads and to obtain counts per gene model locus in TIL11.

Differential Gene Expression Analysis and Functional Annotation

The reads were put into the EdgeR platform (Robinson et al. 2010), followed by removing low-count loci. All libraries were then normalized. A multidimensional scaling plot (MDS) was generated to assess the variation between replicates, followed by clustering of the 24 libraries. Bioreps were clustered together using EdgeR log-fold change analysis to conduct differential gene expression analysis. The differentially expressed genes (DEGs) were then annotated and aligned with the reference genome. The Protein family (Pfam) database, which is commonly used for the functional annotation of proteins (Bateman 2004; Mistry et al. 2020), was utilized for this process. The Emboss transeq tool was used to convert predicted transcripts from RNA-seq analysis into protein sequences. The hmmer3 tool was then used to align protein

sequences with the Pfam motifs, setting the E parameter to ≤ 1e-5. The Pfam database was used to scan predicted proteins for the presence of conserved protein domains. The criteria for selecting good expression candidates were based on: 1) EdgeR log-fold change analysis, where a log-fold change (logFC) value of the two NILs and TIL11 should be at least 1, 2) High-quality expression differences should have a P-value of 0.05 for the library demonstrating the fold-change value of at least 1 (P-value ≤ 0.05), and 3) The DEGs should be upregulated and encode Pfam domains associated with disease resistance functions.

cDNA Synthesis and Quantitative RT-PCR Analysis

A total of 10 µl of RNA samples were used for cDNA synthesis via the Affinity Script Multiple Temperature Reverse Transcriptase cDNA synthesis kit, following the manufacturer's instructions (Agilent Cat # 200436). The resulting cDNAs were then quantified using a Nanodrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA).

Based on RNA-seq analysis, four genes were upregulated in the two NILs and TIL11 in response to *U. maydis*. To validate expression of the four genes quantitative real time PCR (qRT-PCR) was performed for each gene. Gene-specific forward and reverse primer sets were designed utilizing Primer-5 software. qRT-PCR experiments were carried out on a BioRad CFX96 Real-Time PCR System (Bio-Rad, Hercules, CA, USA), employing a ninety-six-well plate format, as directed by the manufacturer. Three biological replicates were conducted for each treated and control sample, with a housekeeping gene (ZmActin) and a negative control. ZmActin was selected as the reference gene because it was stably expressed in all cells (Chen et al. 2019). qRT-PCR was performed using a 25 µl reaction mix containing 2 µl of cDNA, 1 µl of each 10,000 nM forward and reverse primer, 8.5 µl sterile water, and 12.5 µl iQ SYBR Green Supermix (Bio-Rad Cat No. 170-8880). For a negative control, water was added in the master

mix instead of cDNA. The standard thermocycling program included three steps: (1) Template denaturation (a cycle of 95°C for 3 min), (2) Template amplification and quantification (40 cycles at 95°C for 15 seconds, 60°C for 1 min and 1 cycle of 72°C for 10 minutes), and (3) Melt curve analysis (91 cycles at 50°C- 95°C for 30 sec). The relative expressions for each sample were calculated using the 2^(-ΔΔCT) method.

RESULTS

Phenotypic Evaluation of TIL11, NIL1, NIL2, and B73

To determine which genotypes were resistant to *U. maydis*, TIL11, the two NILs, and maize inbred line B73 were observed daily after inoculation for 21 days. The two NILs and TIL11 demonstrated a high level of resistance to *U. maydis* since most of the lines only displayed chlorotic symptoms (scale-1C) and anthocyanin pigmentation (scale- 1A) (**Figure 3.1A**). Only a few NILs exhibited minor leaf galls (scale-2) that persisted from 7 dpi until 21 dpi, which showed their resistance to *U. maydis*. Conversely, B73 began to show symptoms of minor leaf galls (scale-2) 14 days after inoculation (**Figure 3.1B**). These galls were noticeable when the leaf blade was touched, and with time, the lesions grew larger and were accompanied by major stem galls (scale-3 rating) and basal galls (scale-4), leading to the death of some plants (scale-5). The results implied that B73 was higly susceptible to *U. maydis* infection. While mock-inoculated TIL11, B73 and the two NILs did not show any infection.

Differential Gene Expression Analysis in the 3.9 Mbp Region of TIL11, Two NILs and B73

Differential gene expression analysis was used to identify genes that are differentially expressed among the four genotypes. The goal of this study was to identify genes that contribute to the resistance observed for TIL11, and the two NILs against *U. maydis*. Twenty-four genes

were identified in the 3.9 Mbp teosinte introgressed region of chromosome 9 (data not shown). Four (no Pfam, gene 1, gene 2, and gene 3) of the 24 genes were upregulated in all four genotypes, with a logFC value greater than 1 in TIL11 and the two NILs, but less than 1 in B73. Additionally, gene 2 was found to be upregulated with a logFC value greater than 1 in TIL11, less than 1 in NIL1 but downregulated in B73. One gene was significantly upregulated in both TIL11 and the NILs with a logFC value greater than 1 (P-value <0.05). The upregulation of these genes in TIL11 and NILs suggest a role in resistance to *U. maydis*.

One gene was upregulated in TIL11, B73, and NIL2, but downregulated in NIL1. However, the logFC value was less than 1 in TIL11 and the two NILs, while it was higher than 1 in B73. Similarly, another gene was upregulated in TIL11 and NIL1, but downregulated in NIL2 and B73. This gene had a logFC value less than 1 for TIL11 and the two NILs, suggesting it may not be a strong candidate gene. Another gene was upregulated in TIL11 and NIL2 but downregulated in B73 and NIL1. However, the logFC value was lower than 1, indicating it may not be a good candidate gene. Two genes were upregulated in TIL11 and downregulated in both NILs and B73. However, both genes did not have a logFC value greater than or equal to 1 in TIL11, suggesting they may not be strong candidate genes.

Four genes were downregulated in TIL11 and the two NILs with logFC values equal to or greater than 1 (P-value <0.05). These same genes were upregulated in B73, suggesting they may be good candidate genes. Eight genes were downregulated in all four genotypes. For two of the gene's, logFC values were greater than 1 in TIL11 and the NILs, while logFC values for the other genes were lower than 1 in TIL11 and the two NILs when compared to B73.

Lastly, one gene was downregulated in TIL11 but upregulated in B73 and the NILs. However, the logFC for this gene was lower than 1 in TIL11 indicating this may not be a good candidate gene.

In summary, six genes were identified as potential candidates for disease resistance to *U. maydis*. These genes were upregulated in TIL11, and two NILs, (data not shown) with logFC values greater than 1, indicating their robustness as candidates for *U. maydis* resistance. When a gene is upregulated with a logFC value of 1 or greater than 1, it suggests a significant increase in gene expression in resistant TIL11 and the two NILs as compared to B73. This implies that TIL11 and the two NILs may have a stronger and more effective immune response to the pathogen, resulting in higher expression of genes related to immunity and defense. Additionally, four genes were significantly downregulated in TIL11 and the two NILs, with logFC values greater than 1 (P-value <0.05). These genes may play a role in the mechanism that confers resistance in TIL11 and two NILs, and susceptibility in B73.

Protein Family Based Functional Annotation

Functional annotation of the 24 genes was performed using the Pfam database, which is based on the assumption that conserved protein domains indicate functional similarity. This database can identify known protein domains in newly sequenced proteins, even if the proteins have not been functionally characterized. Among the 24 DEGs, only 20 genes had specific functions associated with protein domains, as determined through Pfam-based annotation (see **Table 3.3**).

Eight of the 20 genes were associated with plant disease resistance, pathogenesis, or defense genes, and they encoded Pfam domains. The remaining 12 genes did not have Pfam

domains associated with disease resistance, pathogenesis related, and defense genes. Conversely, the remaining four genes did not have any identifiable protein domains (**Table 3.3**).

Selection of Best Candidate Genes for *Ustilago maydis* Resistance

Four of the 24 genes were identified as potential candidates that may contribute to resistance against *U. maydis* in TIL11 and the two NILs (**Figure 3.2**). Three of these genes, gene 1, gene 2, and gene 3, were selected based on three criteria:1) significant upregulation compared to B73 (P-value <0.05), 2) a logFC value greater than 1, and 3) association with plant disease resistance as indicated by the presence of Pfam domains PK_Tyr_Ser-Thr; Pkinase, LTP_2; Tryp_Alpha_Amyl, and TPR_1. The gene corresponding to no Pfam was selected as a potential candidate for *U. maydis* resistance despite lacking a Pfam domain because it was upregulated with a logFC value greater than 1 in both TIL11 and the two NILs. The significantly higher expression of the four genes in TIL11 and the NILs compared to B73 suggests that they may provide a selective advantage contributing to the resistance phenotype. However, it is worth noting that gene 3 had a large standard error for the TIL11 fold change. To validate the expression of these four genes, qRT-PCR was performed.

Validation of Candidate Genes with Quantitative RT-PCR Analysis

Quantitative real-time PCR experiments were conducted to confirm the DEGs associated with the resistance to *U. maydis* in maize plants by comparative transcriptome analysis. Four DEGs based on their logFC values and Pfam domains associated in other system with disease resistance functions were selected to investigate their relative expression in response to pathogen inoculation. The delta-delta Ct method was used to calculate the fold change of transcript levels

in the inoculated samples with respect to the transcript levels in mock-inoculated B73, TIL11 and the two NILs.

Relative expression levels of four genes via qRT-PCR assays are shown in **Figure 3.3**. The graph showed that relative expression levels of four tested genes (No Pfam, gene 1, gene 2, and gene 3) were significantly higher in the two NILs and TIL11 compared to B73, at 12 hpi. The qRT-PCR experiments confirmed the RNA-seq data and demonstrated that the selected genes could be crucial for *U. maydis* resistance in maize plants. The findings suggest that these genes could be potential targets for enhancing the resistance of maize plants to *U. maydis*.

DISCUSSION

The aim of this study was to identify candidate genes involved in resistance to *U. maydis*. To achieve this goal, transcriptome analysis of TIL11, the two NILs, and B73 was conducted to obtain valuable information about the resistance mechanism to *U. maydis* 12 hrs post inoculation. Twenty-four DEGs were identified across the four genotypes. To select the best candidate genes, we established criteria that included significant upregulation in TIL11 with a logFC value greater than 1, and genes that encode disease resistance Pfam domains. Three (gene 1, gene 3, and gene 2) of the 24 differentially expressed genes met these criteria. Pfam-based annotation identified three genes associated with disease resistance, pathogenesis related or defense functions that include PK_Tyr_Ser-Thr-Pkinase, TPR_1, and LTP_2; Tryp_Alpha_Amyl. A gene with no functional domain (no Pfam) was also selected for further functional characterization as it was significantly upregulated in TIL11 compared to B73.

Gene 1 which encodes a PK_Tyr_Ser-Thr/Pkinase, belongs to the protein kinase superfamily and was upregulated in TIL11 and the two NILs compared to B73. Recent studies have demonstrated the crucial role of tyrosine phosphorylation in plant development and

immunity. Dual-specificity protein kinases in plants, which possess both tyrosine and serine-threonine kinase activity, are involved in these physiological processes. Sugano et al. (2018) showed that Broad-Spectrum Resistance 1 (BSR1), a non-receptor dual-specificity kinase, requires both tyrosine and serine/threonine kinase activities for normal functioning in resistance to multiple pathogens. Tyrosine phosphorylation of BSR1 is critical for proper protein localization in rice cells and plays a crucial role in BSR1-mediated resistance to Rice Blast caused by the fungus *Pyricularia oryzae* and also to bacterial leaf blight caused by *Xanthomonas oryzae* pv. *oryzae*. The authors found that protein kinase activity is essential for resistance to Rice Blast and leaf blight in BSR1-overexpressing plants. Based on these findings, gene 1 (PK_Tyr_Ser-Thr/Pkinase) could be a strong candidate for resistance to *U. maydis*.

Gene 2 which encodes a LTP_2/Tryp alpha amyl, was significantly upregulated in TIL11 and NIL1. Lipid transfer proteins, which belong to the pathogenesis-related protein 14 family, have been reported to inhibit fungal or bacterial growth and act as positive regulators in plant disease resistance (Buhot et al. 2001). Wei and Zhong (2014) demonstrated that some LTP genes showed delayed expression patterns after *U. maydis* infection, specifically LTP_2, with upregulated expression levels. In contrast, our study found that these genes were upregulated at an early time point, specifically 12 hours post-inoculation. Moreover, LTP_2 expression in *Arabidopsis* is induced by the necrotrophic fungus *Botrytis cinerea* and soil bacterium *Agrobacterium tumefaciens* (Chassot et al. 2007), suggesting that LTP_2 contributes to pathogen resistance by maintaining the integrity between the cuticle and the cell wall and is a potential candidate for resistance to *U maydis.*

Tryp_Alpha_Amyl are classified as serine protease inhibitors (SPIs) (Grosse-Holz and van der Hoorn 2016). In plants, SPIs play an important role in regulating infection processes by

targeting proteases released by pathogens. Habib and Fazili (2007) noted that plants increase their expression of SPIs in response to pathogen attack to impede the growth of microorganisms. For example, a trypsin inhibitor from *Vicia faba* can inhibit mycelial growth from a range of fungi, indicating its broad-spectrum inhibitory capacity (Ye et al. 2001). Similarly, a study by Pekkarinen et al. (2007) showed that all three SPIs from barley (subtilisin inhibitor 2, alpha-amylase/subtilisin inhibitor, and trypsin inhibitor) could inhibit various serine proteases from the fungus *Fusarium culmorum*.

Gene 3, which encodes TPR_1, was detected in all four genotypes but showed significant upregulation in NIL1, NIL2, and TIL11 when compared to B73. Proteins containing TPRs are important determinants in signal transduction pathways (Schapire et al. 2006). A recent study by Yang et al. (2019) found that the functional chitinase *MoChia1* is required for the growth and development of *P. oryzae* and it binds chitin to suppress the chitin-triggered plant immune response. However, TPR_1 in rice interacts and binds MoChia1 in the rice apoplast, which allows the accumulation of free chitin and re-establishes the immune response. The expression of TPR1 in rice leaves was significantly elevated following inoculation with *M. oryzae*, indicating TPR1 expression in rice is induced by infection. These findings suggest that TPR_1 plays a positive role in disease resistance to *P. oryzae*. Similarly, SNAPs (Soluble NSF-Attachment Proteins) have been associated with disease resistance in plants (Matsye et al. 2012; Cook et al. 2012) due to their TPR_1 domain. Lakhssassi et al. (2017) characterized the SNAP subfamily of TPR-containing proteins and demonstrated through sequence analysis that SNAP11 contributes to additive resistance to soybean cyst nematode (SCN). GmSNAP18 has also been reported to mediate resistance to SCN (Cook et al. 2012). Therefore, gene 3 (TPR_1) may be a potential candidate for resistance to *U maydis*.

A multidimensional scaling plot was used to assess the similarity or dissimilarity between biological replicates (Tzeng et al. 2008). Out of the three biological replicates, only two were clustered together for both control and treated conditions for all four genotypes. This might raise concerns about the reproducibility and reliability of the RNA-seq data. To address this issue, we performed qRT-PCR on four selected candidate genes (No Pfam, gene 1, gene 2, and gene 3), which validated the results of the RNA-seq analysis and strengthened the conclusion that the observed differences in gene expression are biologically meaningful and not due to technical artifacts. The candidate genes identified were consistent with the RNA-seq outcomes, further supporting these genes are contributing to resistant phenotype.

This study provided valuable insights and a comprehensive list of defense-related transcripts, revealing differences between TIL11, two NILs, and B73. The upregulation of gene 1, gene 2, gene 3, and No Pfam was observed in all four genotypes, indicating a common defense response activated after pathogen inoculation. However, TIL11 and the two NILs exhibited a more potent defense response, suggesting that these genes are associated with resistance to *U. maydis* in these genotypes. The genes differential expression patterns were also validated by qRT-PCR, confirming the consistency with RNA-seq results.

In addition, four genes were found to be significantly downregulated in TIL11 and the two NILs while upregulated in B73. Only two of the genes encode pfam domains associated with resistance or defense, namely, FA_hydroxylase and Cation_ATPase_C, respectively. These genes could be highlighted as promising candidates for further investigation to understand their biological function. However, it is difficult to correlate the downregulation of these genes to be associated directly or indirectly with plant defenses.

This study identified four genes in the 3.9-Mbp region in chromosome 9 associated with disease resistance, pathogenesis, and defense genes that were differentially expressed in TIL11, the two NILs and B73, therefore these gene were classified as candidate genes for resistance to *U. maydis*. While gene expression analysis is informative, it alone cannot provide proof of the underlying mechanisms of disease resistance. To fully understand the specific roles of these four genes in resistance to *U. maydis*, functional validation is necessary. Gene silencing of upregulated genes and overexpressing the downregulated genes would aid in investigating the phenotypic changes resulting from alterations in gene expression following *U. maydis* inoculation.

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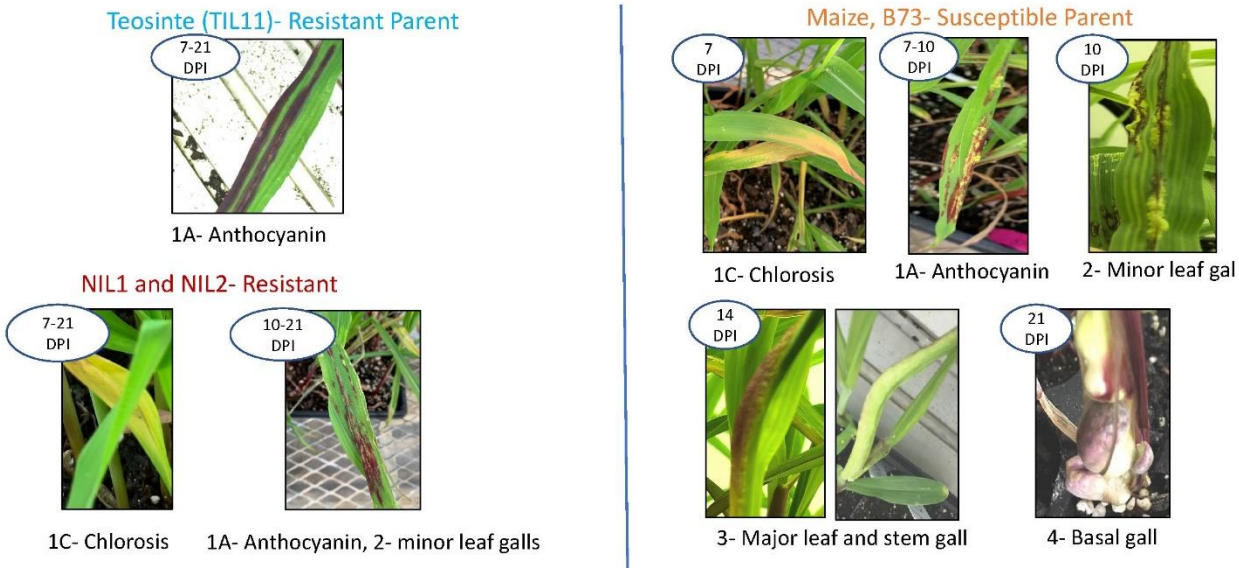

Figure 3.1 A **Figure 3.1 B**

Figure 3.1. Phenotypic evaluation of TIL11, NIL1, NIL2 and B73 seedlings inoculated with *Ustilago maydis*. A disease rating scale of (0-5) was used to assess the disease severity at 7, 10 14 and 21 days post inoculation (dpi). A resistance reaction comprised of scale of 0-2, where 0- completely healthy, 1C- chlorosis, 1A- anthocyanin, 2- minor leaf galls, whereas a susceptible reaction comprised of a rating scale from 3-5, where 3- major leaf and stem galls, 4- basal galls, and 5- death of the plant. **Figure 3.1 A:** Teosinte only developed anthocyanin 7-21 dpi (1A), NIL1 only developed chlorosis 7-21 dpi (1C), and NIL2 only developed anthocyanin and minor leaf galls 7-21 dpi (1A and 2). These results indicate Teosinte, NIL1 and NIL2 are resistant to *U. maydis*. **Figure 3.1 B:** B73 developed chlorosis (1C), anthocyanin (1A) and minor leaf galls (2), major leaf and stem galls (3), basal galls (4), at 7, 10, 14, and 21 dpi, respectively. The scale of 5 represents death of the plant indicating B73 is susceptible to *U. maydis*.

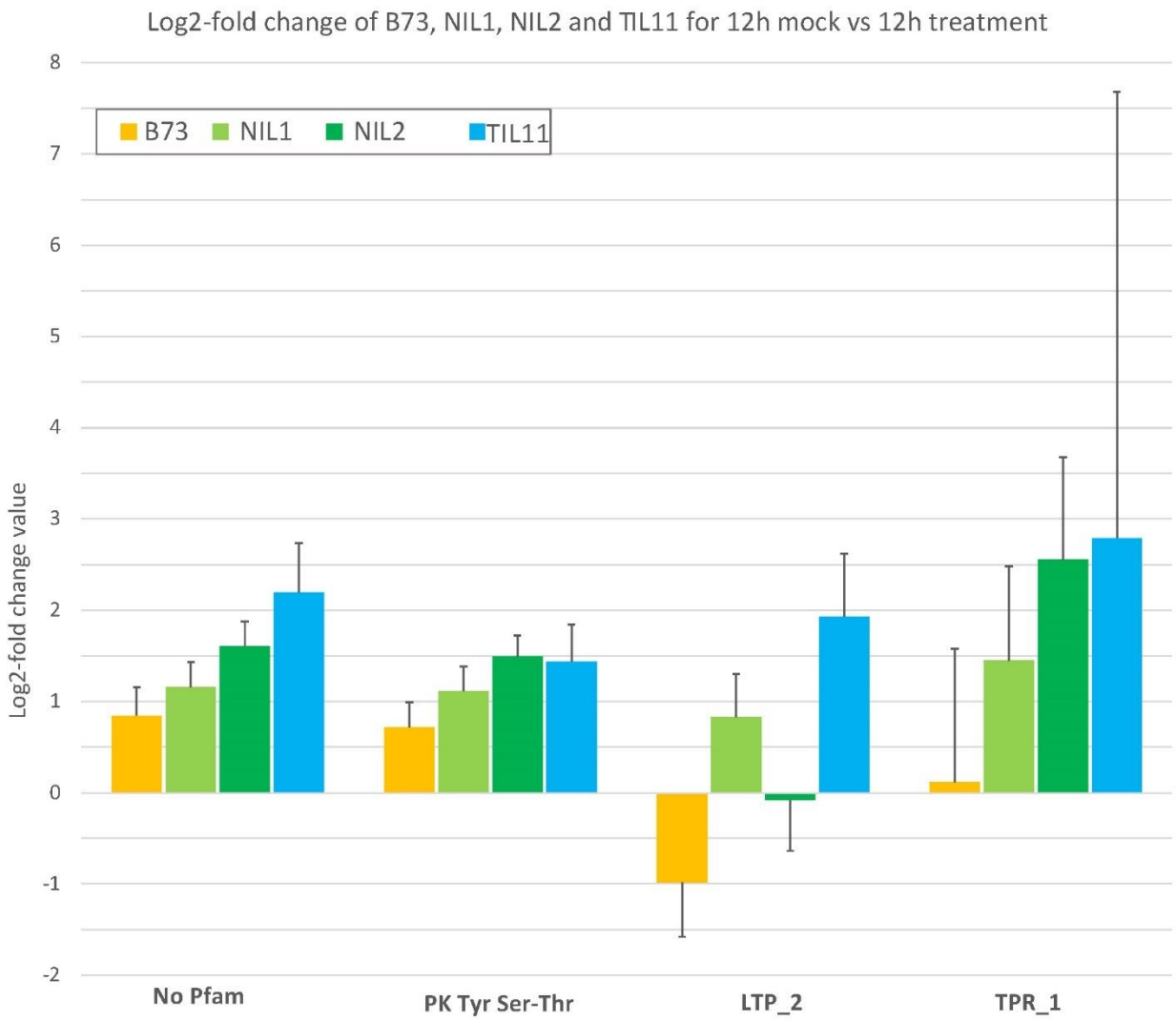

Figure 3.2. Expression levels of four candidate genes in the 3.9 Mbp region of chromosome 9 in TIL11, NIL1, and NIL2 inoculated with *Ustilago maydis*. The x-axis represents four genes encoding No Pfam, PK_Tyr_Ser-Thr, LTP_2, and TPR_1 domains. The y-axis shows the log fold change values and the legend indicates the four genotypes.

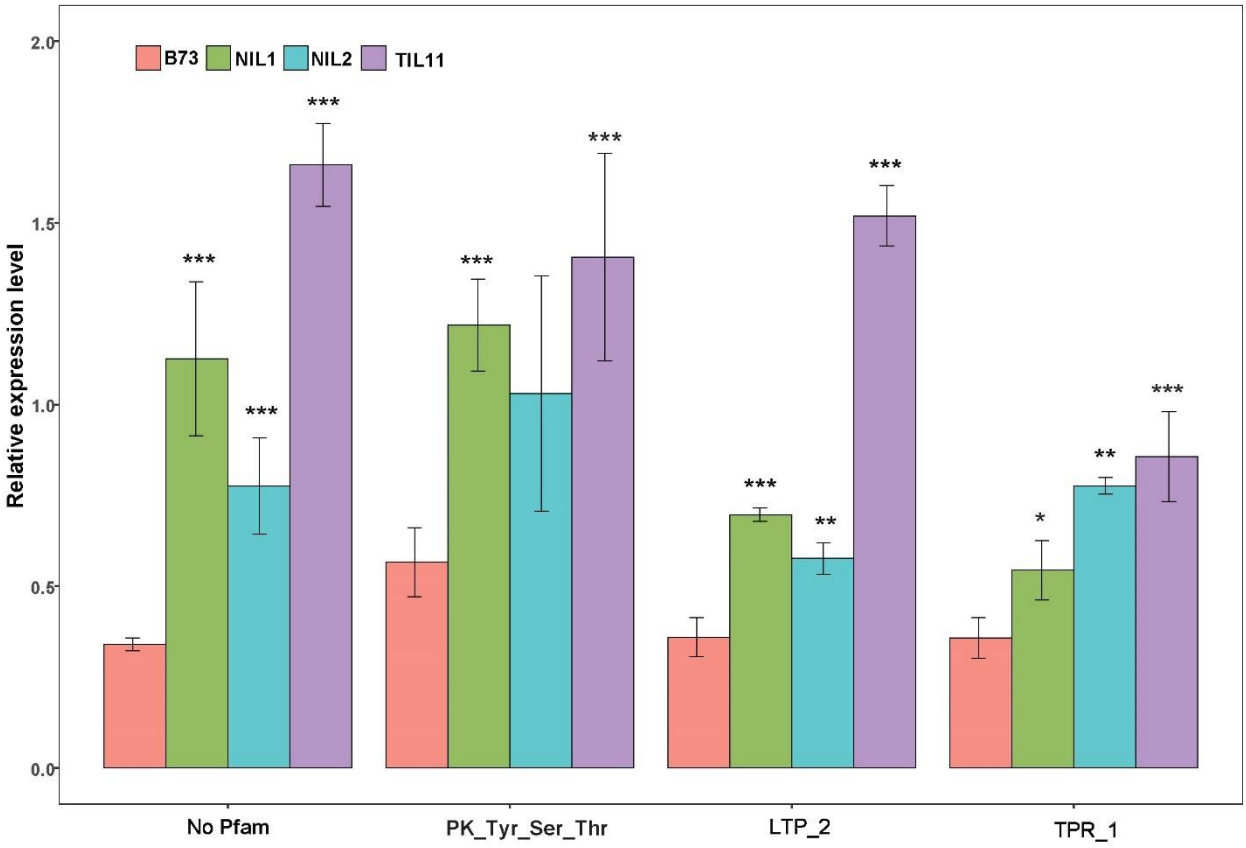

Figure 3.3. Relative expression and validation of four candidate genes in the 3.9 Mbp region of chromosome 9 in TIL11, NIL1 and NIL2 using quantitative real time PCR. qRT-PCR analysis of four candidate genes confirmed that the expression levels of four genes: No Pfam, gene 1, gene 2, and gene 3 were significantly higher in two NILs and TIL11 compared to B73. Delta delta Ct method ($2^{-\Delta\Delta Ct}$) was used to calculate the fold change of transcript levels in the *U. maydis* inoculated samples with respect to the transcript levels in mock-inoculated B73, TIL11 and the two NILs (NIL1 and NIL2). Data are shown as mean±standard errors ($p \leq 0.05$ *; $p \leq 0.01$ **; $p \leq 0.001$***; Student's t-test). Mean values standard errors (se) from three technical replicates of qRT-PCR assays are shown.

CHAPTER 4

FUNCTIONAL CHARACTERIZATION OF GENES ASSOCIATED WITH RESISTANCE TO *USTILAGO MAYDIS* IN TEOSINTE AND TWO MAIZE-TEOSINTE NEAR-ISOGENIC LINES WITH VIRUS-INDUCED GENE SILENCING[3]

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ABSTRACT

Whole genome and RNA sequencing generate a large repertoire of candidate genes in plants associated with specific agronomic traits, including disease resistance. Rapid and high-throughput functional genomics approaches are needed to validate the biological function of these candidate genes. One tool that expedites the validation of gene function in crops genetically difficult to transform is Virus-induced gene silencing (VIGS). In this study, four candidate genes: 1. gene 1 (PK_Tyr_Ser-Thr/Pkinase), 2. gene 2 (LTP_2/Tryp_alpha_amyl), 3. gene 3 (TPR_1), and 4. gene 4 (No Pfam) that encode pfam domains associated with disease resistance/pathogenesis/defense (1-3) and significantly upregulated in TIL11 *(*teosinte inbred line; *Zea mays* ssp. *parviglumis*) and two near-isogenic lines (NILs) in response to *Ustilago maydis* were selected for VIGS. In order to validate the function of these four genes, the VIGS approach using FoMV (*Foxtail mosaic virus*) vector system and agro-inoculation method was used. Gene fragments of the four genes were inserted into an infectious FoMV viral genome that was cloned into the binary T-DNA plasmid vector pCAMBIA1380. The resulting plasmid constructs were then transformed into *Agrobacterium tumefaciens* strain GV3101. *Agrobacterium*-based inoculation was performed to introduce four viral vectors with four target genes (1. FoMV:PK_Tyr_Ser-Thr, 2. FoMV:LTP_2, 3. FoMV:TPR_1, and 4. FoMV:No Pfam) and one empty vector (FoMV:EV) independently in seedlings of TIL11, NIL1, and NIL2. A total of 540, 4-6 days old seedlings were inoculated near the coleoptile node. Viral symptoms were observed on the leaves as the recombinant virus replicate and systemically spreads throughout the plant. Twelve to fourteen days post virus inoculation, the seedlings were inoculated with *U. maydis.* Leaf tissue samples were collected at 0 hpi, 12 hpi, 72 hpi, and 8 dpi. qRT-PCR analysis and phenotypic evaluation was performed. The time course study revealed that the expression

levels of all four genes were significantly reduced, ultimately increasing the susceptibility of TIL11 and the two NILs to *U. maydis,* as evidenced by the galls on leaves, stems, and basal parts of the plants. This suggests the four candidate genes in the 3.9 Mbp region likely contribute to the resistance observed for TIL11 and the two NILs. Resistance to *U. maydis* is quantitative, therefore the candidate genes are likely contributing incrementally to the resistance. This study also demonstrated VIGS is a viable method for gene validation in teosinte and maize.

Keywords: Teosinte Inbred Line, Near-isogenic lines, *Ustilago maydis*, qRT-PCR, Virus-induced gene silencing

INTRODUCTION

Virus-induced gene silencing (VIGS) is a post- transcriptional gene silencing technique that targets and leads to the degradation of viral RNAs (Vance and Vaucheret 2001; Baulcombe 2004). It exploits the natural defense mechanisms plants employ to protect against invading viruses (Voinnet 2002). Post-transcriptional gene silencing in plants depends on a relatively high degree of nucleotide homology between RNA transcripts and target endogenous gene sequences (van den Boogaart et al. 1998; Ding 2000). Plants infected by the recombinant virus-carrying sequence of a host gene generate dsRNAs, which are processed by Dicer-like proteins to produce siRNAs, and the latter triggers the silencing of the endogenous gene. VIGS is a valuable biotechnological tool for the genetic and functional characterization of genes in plants (Pasin et al. 2019; Zaidi and Mansoor 2017). Generating stably transformed transgenic plants to assess gene function is a lengthy multi-layered process. As an alternative, foreign gene expression in plants is often performed by the transient transformation of cells or tissues (Orzaez et al. 2006).

Plant viruses provide surprisingly versatile technology platforms enabling the expression of a wide array of coding and non-coding sequences in plants (Pasin et al. 2019). Viruses engineered to carry heterologous open reading frames (ORFs) can express the encoded proteins. Viruses that carry fragments of plant genes in sense and antisense orientation cause VIGS of the targeted sequence or micro RNA inserts can initiate silencing with high specificity (Mei et al. 2019). Plant viral vectors have many unique advantages over other technologies, including speed and the ability to silence or overexpress genes in different genetic backgrounds. They can knock down the expression of a single gene, a gene family, or a combination of distinct genes. Due to these advantages, viral vectors have been developed and used in dicot and monocot plants. For monocots, utill now, at least eight different viruses have been used to develop into viral vectors, including *Barley stripe mosaic virus* (BSMV) (Lee et al. 2012; Scofield et al. 2005), *Brome mosaic virus* (BMV) (Ding et al. 2006), *Cymbidium mosaic virus* (CymMV) (Hsieh et al. 2013), *Rice tungro bacilliform virus* (RTBV) (Purkayastha et al. 2010), *Wheat streak mosaic virus* (WSMV) (Choi et al. 2000; Tatineni et al. 2011), *Bamboo mosaic virus* (BaMV) together with its associated satellite RNA (Liou et al. 2014), *Cucumber mosaic virus* (CMV) (Wang et al. 2016), *Barley yellow striate mosaic virus* (BYSMV) (Gao et al. 2019), and *Foxtail mosaic virus* (FoMV) (Bouton et al. 2018; Liu et al. 2016; Mei and Whitham 2018; Mei et al. 2016). In addition, seven of the virus vectors are designed for VIGS applications (BSMV, BMV, CymMV, RTBV, BaMV, CMV, and FoMV), and four can be used for systemic gene expression (BSMV, BYSMV, FoMV, and WSMV).

FoMV viral vector system has the potential to offer conventional genetic and transgenic plant approaches for identifying gene functions. It has been demonstrated that FoMV produces systemic infection and is able to induce VIGS in maize (Liu et al. 2016; Mei and Whitham 2018;

Mei et al. 2016) or express proteins (Bouton et al. 2018). The ability of FoMV to infect maize and other monocots makes it a candidate for viral vector development (Paulsen and Niblett 1977). FoMV has a broad host range including 56 species of Poaceae (e.g. maize, sorghum, rice, barley, and green foxtail and at least 35 dicot species (e.g. soybean and tobacco) (Paulsen and Niblett 1977). Mei et al. 2016 confirmed that at least nine maize inbred lines, sorghum, and green foxtail could be infected by an FoMV isolate. Bouton et al. 2018 and Mei et al. 2019 confirmed the FoMV vector to express proteins in maize. Thus, the successful demonstration of the FoMV vector for gene silencing and overexpression in corn is expected to readily translate into a useful functional genomics platform for research and crop improvement.

FoMV is a member of the genus *Potexvirus*, which has a single-stranded, positive-sense genomic RNA with a 59-methylguanosine cap, a 39-polyadenylated tail, and five major open reading frames (ORFs) and a unique 5A gene (Robertson et al. 2000; Bruun-Rasmussen et al. 2008). The genome structure of FoMV is similar to potato virus X (PVX), a member of the potexviruses. These viruses typically contain five major open reading frames (ORF) (Bruun-Rasmussen et al. 2008; Robertson et al. 2000). ORF1 encodes the RNA dependent RNA polymerase (RdRp), which is necessary for viral RNA replication and subgenomic messenger RNA (sgRNA) synthesis (Draghici and Varrelmann 2009). The overlapping ORFs 2, 3, and 4 are known as the triple gene block (TGB) with functions in virus movement and suppression of host defense (Verchot-Lubicz 2005). ORF5 encodes the coat protein (CP), which is indispensable for virus assembly and cell-to-cell movement (Cruz et al. 1998). In addition to the five ORFs, the FoMV genome has a unique ORF5A that initiates 144 nucleotides upstream of the CP, but it is not required for replication or systemic infection of *Nicotiana benthamiana* on barley (Robertson et al. 2000). FoMV has been modified as an RNAi suppressor-dependent expression vector by

deleting its CP and triple gene block genetic segments (Liu and Kearney 2010). FoMV was previously developed into both a VIGS and VOX (virus induced gene overexpression) vector for maize by incorporating an infectious clone onto a T-DNA plasmid backbone (Mei et al. 2016; Bouton et al. 2018; Mei et al. 2019). The viral genome was modified for VIGS applications by adding a cloning site (MCS1) downstream of the CP (**Figure 4.1**) (Mei et al. 2016). For VOX and virus enabled gene editing applications, the CP promoter was duplicated, and a second cloning site (MCS2) was added to enable the insertion of sequences of interest between ORF 4 and the CP (**Figure 4.1**) (Mei et al. 2019). The FoMV vector containing both MCS1 and MCS2 with no inserts is FoMV empty vector (FoMV-EV). T-DNA plasmids carrying infectious cDNA of these recombinant viruses have been transformed into *Agrobacterium tumefaciens* strain GV3101 that is well known to transfer T-DNA to monocotyledonous species, including maize (Marks et al. 1989). Several agroinjection studies have used the C58 strains or its derivative GV3101 (Grimsley et al. 1988; Martin and Rybicki 2000). Current methods to initiate virus infections in plants consist of particle bombardment, mechanical inoculations of in vitro RNA transcripts or DNA clones, vascular puncture inoculation, and *Agrobacterium tumefaciens* inoculation (agroinoculation) (Cody and Scholthof 2019; Redinbaugh et al. 2001; Scholthof 1993; Scholthof 1995; Scholthof et al. 1999; Zhang et al. 2017; Vaghchhipawala et al. 2011; Mei et al. 2016; Wang et al. 2016; Zhu et al. 2017; Liu et al. 2016; Mei and Whitham 2018). These inoculation methods have advantages and disadvantages, which include cost, the need for specialized equipment, and feasibility within a given plant-virus system. Therefore the selection of the most feasible inoculation approach is of prime importance.

Maize is one of the most important cereal crops globally, widely used for various purposes, such as food, feed, fodder, and biofuel. However, corn smut, caused by the fungus

Ustilago maydis, poses a significant threat to maize production, particularly in developing countries, and practical and sustainable management practices to control the disease are currently lacking. Modern maize cultivars have a limited genetic base and have lost some of their natural defenses, making them susceptible to *U. maydis* infection. Therefore, there is an urgent need to identify and characterize resistance to this disease. Previous research has demonstrated that disease resistance, pathogenesis related and defense genes can be introduced from wild ancestors to combat various plant diseases (Liu et al. 2010; Christov 2012; Nenova et al. 2014). In this regard, teosinte, a wild ancestor of maize is resiatant to several pathogens (Singh et al. 2021; Sahoo et al. 2021). Of the several teosintes, *Zea mays* ssp. *parviglumis*, also known as Teosinte inbred line (TIL11), represents a promising candidate for breeding programs due to its compatibility with inbred maize lines and its potential ability to exhibit resistance to *U. maydis*.

In the previous work, phenotypic evaluation identified two near-isogenic lines (NILs) in a maize (B73) x teosinte (TIL11) introgression population that are resistant to *U. maydis* (Chavan and Smith 2014). B73 is the susceptible parent, while TIL11 is the resistant parent. Genotypic analysis of the two resistant NILs identified a 3.9 Mbp teosinte introgressed region on the short arm of chromosome 9 that was present only in the two resistant NILs and absent in the susceptible NILs. These findings strongly suggested that the observed resistance in NIL1 and NIL2 is likely due to the introgressed teosinte genomic segment inherited from the resistant TIL11 parent.

Furthermore, a comparative analysis of the genomes of TIL11 and B73 was conducted, which resulted in the discovery of potential candidate genes in the 3.9-Mbp region of chromosome 9, as detailed in Chapter 2. RNA-seq analysis was employed to determine the expression levels of the candidate genes within the 3.9-Mbp region. RNA-seq analysis (Chapter

3), identified four genes significantly expressed in the two NILs and TIL11 in response to *U. maydis*. These genes encode pfam domains associated with plant disease resistance, pathogenesis related and defense functions (**Table 4.1)**.

In this study, we aim to develop a FoMV vector system capable of establishing viral infection in plants and carrying foreign gene insertions homologous to endogenous host genes targeted for RNA silencing. Although VIGS has been reported in several monocots, including maize inbred lines, this study reports the use of VIGS for the first time in NIL1, NIL2, and TIL11. A preliminary experiment was carried out using three inoculation methods to select an efficient method by which FoMV infects both NILs to successfully deliver the FoMV viral vector into plant tissues. Agro-inoculation, rub inoculation, and vascular puncture inoculation methods were tested. The agro-inoculation method was more efficient and less time-consuming than the other two methods. The specific objectives of this study were to: 1. validate the function of four candidate genes gene 1 (encode pfam domain PK_Tyr_Ser-Thr/Pkinase), gene 2 (encode pfam domain LTP_2/Tryp_alpha_amyl), gene 3 (encode pfam domain TPR_1), and gene 4 with no Pfam using virus-induced gene silencing transient assays in TIL11, NIL1 and NIL2.

Hypothesis

VIGS and the FoMV vector system will successfully silence candidate genes in the 3.9 Mbp region of chromosome 9 in TIL11 and the two NILs resulting in increased susceptibility of these lines to *U. maydis*, validating the function of one or more of the candidate genes in resistance.

MATERIALS AND METHODS

Generation of *Foxtail mosaic virus* Gene Silencing Construct and Sequencing of *Foxtail mosaic virus* Empty Vector

Foxtail mosaic virus empty vector **(**FoMV: EV) stock from the -80°C freezer was streaked onto Luria Bertani (LB) plates containing Kanamycin and incubated overnight at 37°C. Single colonies were selected and grown in LB broth for 24 hours at 37°C with shaking at 225 rpm. Plasmid preps were performed following the manufacturer's protocol and the resulting concentrations of 117 ng/µl and 114 ng/µl were determined using a spectrophotometer. A master mix was then prepared by combining 7.9 µl of H_2O, 2 µl of cut smart, 1 µl of XbaI, and 0.5 µl of PacI, with the resulting mixture of 11.4 µl. A 5.7 µl aliquot of this master mix was added to each of the two Eppendorf tubes that contain 4.3 µl of plasmid DNA. The samples were then incubated at 37°C for 30 minutes. To determine the quality and quantity of the extracted plasmid DNA, 2 µl of FoMV: EV plasmids were run on a 1% agarose gel at 130 volts for 20 minutes. The plasmids were screened for the presence of FoMV: EV using a gel doc without any smearing and degradation. After confirming the presence of the plasmid DNA on the gel, one plasmids DNA sample was sent to Georgia Genomics Facility at UGA for sequencing to confirm its correct nucleotide sequence. This study used FoMV: EV as a vector for inserting the target sequence and as a positive control.

Fragment Selection of Target Gene and Primer Design

Four genes of interest significantly upregulated in TIL11, and the two NILs were selected for virus induced gene silencing based on RNA-Seq analysis. A BLAST search of the transcript sequences for the genes was conducted using a maize genome database to identify the target region of the sequence for silencing and to minimize off-target silencing (Zhou et al. 2018). A

query sequence ranging from 65-80 base pairs (bp) in length was selected for each of the four genes and oligos were designed for use with NEBuilder (New England Biolab). The target sequence was selected for each gene for silencing and the reverse complement of the targets between the 5'-XbaI and 3'-PacI sites was designed to facilitate cloning. The designed oligos for each gene were ordered from Sigma as a single-stranded DNA allowing for the amplified sequence to be inserted into a vector.

Cloning the Selected Fragment of Target Genes into the *Foxtail mosaic virus* Vector

For each of the four target genes, 1 µl of vector and 3 µl of NEB builder were added to separate Eppendorf tubes. Then 2.5 µl of oligos were added to each tube and placed in an incubator for 30 minutes at 50°C. Next, 2 µl of ligated plasmid was transformed into 25 µl of NEB 10 β competent *Escherichia coli* cells using the heat shock method (Krishnan et al. 2015) The resulting four transformations were incubated on ice for 30 minutes, followed by a 30 second heat shock at 42°C and another 4- minute period on ice (Krishnan et al. 2015). Next, 200 µl of NEB outgrowth was added to each tube, which was then placed in a shaker for 1 hour at 37°C with shaking at 225 rpm. Finally, the samples were streaked onto Kanamycin selective LB plates and incubated at 37°C overnight. After incubation for 24 hours, PCR was performed to amplify the fragment in the target region with the gene-specific forward primer FoMV ORF5 and reverse primers pFoMV 3852. A master mix of 660 µl was prepared using 4.4 µl of H_2O, 5 µl of Apex, 0.3 µl of forward primer FoMV ORF5 (TAGTCCCATCCTCTGTACC), and 0.3 µl of reverse primer pFoMV 3852 (GTCCGTAAACCTGCCCATA).

Six colonies were selected for each gene. A total of 15 colonies were selected for each of the four genes, along with a positive and negative control. To each PCR tube, 10 µl of the master mix was added and single colonies were selected by drawing a circle on the plates. Using a

sterilized tip, a small portion of a single colony was picked and placed into the same PCR tubes. FoMV: EV plasmid DNA (0.5 µl) was added for a positive control. The samples were set up in a PCR for 27 cycles for 30 minutes and run on an agarose gel. The resulting colonies were screened for accurate clones by visualizing expected-sized bands on the gel. LB broth (3 ml) was inoculated with appropriate colonies for each of the four genes and incubated at 37°C overnight with shaking at 225 rpm. Three colonies were selected for each of the four genes, and the plasmid DNA sample was extracted from the overnight cultures using the manufacturer's protocol. The concentration of each plasmid DNA sample was measured using a spectrophotometer. Two plasmid DNA samples were selected from each of the genes based on their concentrations as follows: **1)** gene 1 (PK_Tyr_Ser-Thr/Pkinase), plasmid DNA-1 concentration= 392.8 ng/µl, and plasmid DNA-2 concentration= 52.48 ng/µl; **2)** gene 2 (LTP_2/Tryp_alpha_amyl), plasmid DNA-1= 129.69 ng/µl, and plasmid DNA-2= 193.79 ng/µl; **3)** gene 3 (TPR_1) plasmid DNA-1= 197.21 ng/µl and plasmid DNA-2= 41.21 ng/µl; **4)** gene 4 with no Pfam, plasmid DNA 1= 174.97 ng/µl, and plasmid DNA 2= 99.87 ng/µl. Then, 2.5 µl of the forward primer was added to each 12.5 µl of plasmid DNA resuspended in sterile water. A total of eight plasmid DNA samples, including two plasmid DNA samples from the same gene, with a total volume of 15 µl containing 600 ng of plasmid DNA was submitted to the Georgia Genomics Facility at the University of Georgia, for Sanger sequencing. The purpose of the sequencing was to verify the accuracy of the target gene sequences.

Transforming Plasmid DNA into *Agrobacterium* strain GV3101

Using the electroporation method, plasmid DNA with the correct sequence was then transformed into *Agrobacterium tumefaciens* strain GV3101. To prepare for transformations, 900 µl of LB broth was added to five Eppendorf tubes for each of the strains carrying one of the five

plasmid DNA samples. A volume of 1 µl of plasmid DNA was added and mixed with 35 µl of GV3101 in each of the five tubes. The mixture was transferred into an electroporation cuvette, and an electrical pulse was applied to the cells using an electroporator device to introduce the plasmid DNA into the cells. All the samples displayed approximately 2.20 volts and a time of 5.70 sec. Then, 900 µl of LB broth was quickly added to the cuvette between electrodes, properly mixed, and transferred into the new Eppendorf tubes. The transformed cells were incubated in a shaker at 28°C for 2 hours at 250 rpm, and colonies were obtained by streaking the transformed cells onto LB plates. The LB plates were incubated at 28°C for two days. After two days, six individual colonies were picked from each of the five transformed plasmids, and PCR was performed on 30 colonies. A master mix containing 4.4 µl of water, 5 µl of Apex primers, 0.3 µl of forward and reverse primers were prepared to a final volume of 340 µl. Then, 10 µl of the master mix was added to each of the six tubes for the six single colonies. The PCR conditions were as follows: (i) an initial denaturation step at 95°C for 3 minutes, followed by 3 cycles of 98°C for 20 s, 58°C for 20 s, and 72°C for 20 s; (ii) 34 cycles of 72°C for 2 minutes with a final extension step. The PCR products were run on a 1% agarose gel to screen for the presence of an insert in the colonies using a gel doc.

Preparation of Agrobacterium for Agroinfection

A 500 mL volume of LB liquid media with kanamycin (50 µg/mL) or gentamycin (50 µg/mL) antibiotic was prepared a day prior to plant injection (Beernink et al. 2021). Single colonies of the FoMV constructs (**1.** FoMV:PK_Tyr_Ser-Thr/Pkinase, **2.** FoMV:LTP_2/Tryp_alpha_amyl, **3.** FoMV:TPR_1, **4.** FoMV:No Pfam, and **5.** FoMV:EV) were transformed into GV3101 and cultured individually in 30 mL LB broth in small conical flasks. The inoculum was grown in a shaker at 28°C for 24 hours at 225 rpm and then transferred to a

50 mL tube the next day. The bacterial pellet was obtained by centrifugation for 10 minutes at 3500 rpm at room temperature, after which the supernatant was discarded, and the pellet was washed with 5 mL of infiltration buffer by pipetting up and down. The infiltration buffer was prepared by mixing 10 mL of 10 mM $MgCl_2$ and 5 mL of 0.2 mM MES (pH 5.5) in 100 mL H_2O (Krishnan et al. 2015). The bacterial pellet was centrifuged again for an additional 10 minutes at 3500 rpm. The supernatant was discarded, and the pellet was resuspended in 20 mL of induction buffer. The induction buffer was prepared by mixing 200 µl of Acetosyringone (200 mM) in 100 ml of infiltration buffer to enhance the transformation ability of *Agrobacterium* strains (Liu et al. 2016). The 20 mL of the mixed solution was vortexed for 2 minutes and placed in a shaker in a dark room for 4 hours before centrifugation at 3500 rpm. The OD_{600} of the sample was measured with a spectrophotometer and diluted to an OD_{600} of 1 with infiltration buffer (Beernink et al. 2021).

Seedling Preparation and Agro-inoculation of Maize Seedlings

A total of 540 seedlings from the two NILs and TIL11 were grown in peat-based growing soil in small inserts within trays for 7-8 days before the inoculation. Each genotype was inoculated with four inserts, while seedlings inoculated with an empty vector served as controls. Twelve seedlings from each genotype were used for agro-inoculation with each construct and the empty vector. The bacterial suspension with each construct and empty vector was injected into the seedlings 2-3 mm above the coleoptile node using a 25G x 5/8” needle attached to a 1 mL disposable syringe. Seedlings were injected with 200-300 µl of suspension until the suspension filled up the coleoptile or were visible in the whorl (**Figure 4.2)**. Syringes and needles were changed for each construct. The seedlings were transplanted to larger pots when they were 8-9

days old and placed in a growth chamber with a 16-hour day and 8-hour night cycle at 72°F. The seedlings were watered and fertilized once using Osmocote Smart-Release Plant Food Plus.

Inoculation of Teosinte Inbred Lines and Near-Isogenic Lines with *Ustilago maydis*

A wild-type *U. maydis* strain ½ (mating type a_1b_1) and a near-isogenic strain 2/9 (mating type a_2b_2) were used for inoculation. Three PDA plates were streaked with each of the *U. maydis* strains (a_1b_1 and a_2b_2). Single spores were cultured separately in three test tubes for each of the strains. Both strains were grown in potato dextrose broth at 30°C to an OD_{600} of 1.0 (Allen et al. 2011). Cells were suspended in water to a final concentration of 1×10^6 cells/ml. Seedlings that showed viral symptoms were inoculated with *U. maydis* cell suspension culture 12-14 days post-agro-inoculation. The culm of the plants (TIL11 and the two NILs), just above the soil line, was inoculated with ~200-300 µl of cell suspension culture with a final concentration of 1×10^6 cells/ml (Chavan and Smith 2014).

Phenotypic evaluation and qRT-PCR of Teosinte Inbred Lines and Near-Isogenic Lines Inoculated with *Ustilago maydis*

For phenotype confirmation, TIL11 and the two NILs were screened for FoMV infection 12-14 days post-inoculation. Following FoMV infection, TIL11 and the two NILs inoculated with *U. maydis* were screened for galls on leaves, stems, or basal part of the plant 7-21 days post *U. maydis* inoculation. Seedling disease severity was visually assessed using a 0-5 disease rating scale (Allen et al. 2011; Smith and Chavan 2014) where 0 = completely healthy, 1C = chlorosis, 1A = anthocyanin pigmentation, 2 = minor leaf galls, 3 = major leaf and stem galls, 4 = basal gall, and 5 = death of the plant.

qRT-PCR was performed to determine whether the four genes were silenced. Each experiment included three independent samples (biological replicates), each performed in

triplicate (technical replicates). Three seedlings from each of the treated (Agro-inoculated with FoMV constructs and *U. maydis* inoculated) and control (Agroinoculated with empty vector and *U. maydis* inoculated) groups were selected for each time point. Leaf samples (4^{rth} -6^{th} leaf) were collected at 0, 12, 72 hours post-inoculation (hpi), and 8 days post-inoculation (dpi) for RNA extraction. Each leaf was placed in a separate 1.5 ml eppendorf tube and immediately frozen in liquid nitrogen.

Total RNA was extracted from leaves (~70 mg) using Trizol reagent (Thermo Fisher Scientific, Waltham, USA) extraction method according to manufacturer's instructions. The extracted RNA was used as a template to generate cDNA. cDNA synthesis was performed using the Affinity Script Multiple Temperature Reverse Transcriptase cDNA synthesis kit as described by the manufacturer (Agilent Cat. No. 200436). The cDNA reaction was set up with 10 µl of total diluted RNA, 4.7 µl of RNase-free water, 1 µl of oligo (dT) primers, 2 µl of 10X Affinity Script RT Buffer, 0.8 µl of dNTPs, 0.5 µl of RNase block ribonuclease inhibitor, 1 µl of Affinity Script Multiple Temperature RT for a final volume of 20 µl. qRT-PCR was performed using Power SYBR Green PCR Master Mix (Applied Biosystems, Warrington, Cheshire, UK) following the manufacturer's instructions. Primer-5 software was utilized to design gene-specific forward and reverse primers that lie just outside the target sequence that was used to design the construct. The purpose of these primers was to flank the target sequence/construct and ensure that only the intended target is amplified. Zm-Actin was used as an internal control. Each 10 µl quantitative real-time PCR reaction contained a mixture of: 1 µl of cDNA, 0.5 µl of each 10,000 nM forward and reverse primer, 3 µl sterile water and 5 µl iQ SYBR Green Supermix (Bio-Rad Cat No. 170-8880). The standard thermocycling program consisted of: (1) Template denaturation (a cycle of 95°C for 3 min), (2) Template amplification and quantification (40 cycles at 95°C for

15 seconds, 60°C for 1 min and 1 cycle of 72°C for 10 minutes), and (3) Melt curve analysis (91 cycles at 50°C -95°C for 30 sec). The mean relative expression values for TIL11, NIL1 and NIL2 inoculated with FoMV:PK_Tyr_Ser-Thr/Pkinase, FoMV:LTP_2/Tryp_alpha_amyl, FoMV:TPR_1, FoMV:No Pfam, and FoMV:EV, at the four-time points were calculated with the following formula:

$$2^{-\Delta\Delta CT} = 2^{-((CT,Traget-CT,Reference)-(CT,Virus-CT,Reference,Virus))}$$

where:

- ΔΔCT gives the difference between the ΔCT values of the infected and uninfected samples
- CT, Target: the cycle threshold for the target gene in the experimental sample
- CT, Target, Virus: the cycle threshold for the target gene in the experimental sample after virus infection
- CT, Reference: the cycle threshold for the reference gene in the experimental sample
- CT, Reference, Virus: the cycle threshold for the reference gene in the experimental sample after virus infection

RESULTS

Confirmation of Integration of All Four Target Genes into the *Foxtail mosaic virus* Vector

Eight plasmid DNAs were sent for sequencing and at least one plasmid DNA for each gene was in the correct orientation. The purpose of the sequencing was to verify the accuracy of the target gene sequences cloned into the VIGS vector: **1.** gene 1 (PK_Tyr_Ser-Thr/Pkinase), **2.** gene 2 (LTP_2/Tryp_alpha_amyl), **3.** gene 3 (TPR_1), and **4.** gene 4 (No Pfam).

Transformation of Plasmid DNA with Target Gene into *Agrobacterium* Strain GV-3101

The presence of bands in the gel **(Figure 4.3)** confirmed the successful transformation of the four constructs into the *Agrobacterium tumefaciens* strain GV-3101 (1. FoMV:PK_Tyr_Ser-Thr/Pkinase, 2. FoMV:LTP_2/Tryp_alpha_amyl, 3. FoMV:TPR_1, and 4. FoMV:No Pfam). The PCR primers produced an amplicon that spans MCS1. The positive control, FoMV:EV plasmid, exhibited an anticipated amplicon size of 315 bp. Moreover, the PCR amplification of FoMV:PK_Tyr_Ser-Thr/Pkinase yielded four strong bands with an amplicon size of 380 bp, while FoMV:LTP_2/Tryp_alpha_amyl produced two strong bands with an amplicon size of 385 bp. FoMV:TPR_1 resulted in six strong bands with an amplicon size of 386 bp, and FoMV:No Pfam produced two strong bands with an amplicon size of 375 bp. These findings indicate that the *Agrobacterium tumefaciens* transformations carry the plasmid containing the target gene.

***Foxtail mosaic virus* Infection**

FoMV infection was observed in TIL11, as well as in NIL1 and NIL2 seedlings that were inoculated with FoMV:PK_Tyr_Ser-Thr/Pkinase, FoMV:LTP_2/Tryp_alpha_amyl, FoMV:TPR_1, FoMV:No Pfam, and FoMV:EV. After approximately 12 days following agro-injection, the plants started exhibiting distinct phenotypic characteristics on their leaves. Infected seedlings displayed a patchy or mosaic-like pattern on their leaves, with areas of light and dark green (**Figure 4.4)**.

Susceptibility of Agro-inoculated Seedlings

To evaluate the phenotype conferred by the VIGS construct, *U. maydis* inoculation was performed, and disease assessment was conducted at different time intervals (7, 10, 14, and 21 dpi) (**Figure 4.5, left hand side)**. Using a disease rating scale of 0-5, the majority of seedlings for all three genotypes agro-inoculated with FoMV:PK_Tyr_Ser-Thr/Pkinase (**Figure 4.5 A1)**,

FoMV:LTP_2/Tryp_alpha_amyl (**Figure 4.5 B1)**, FoMV:TPR_1 (**Figure 4.5 C1)**, FoMV: No Pfam (**Figure 4.5 D1)**, exhibited high susceptibility to *U. maydis*, with severe leaf galls, stem galls, and basal galls observed within 7-21 days post-inoculation. Conversely, seedlings inoculated with FoMV: EV showed minor symptoms, such as a few chlorotic spots, anthocyanin, and minor leaf galls, indicating resistance (**Figure 4.5)**. These findings suggest that the candidate gene in each construct silenced the internal plant gene resulting in increased susceptibility in the TIL11 and the two NILs and support the idea that the candidate genes are involved in plant defense against *U. maydis*.

Quantitative Real-Time PCR Evaluation

After detecting systemic viral infection in TIL11, the two NILs at the 4-6th leaf stage, an assessment of gene silencing efficiency of the four constructs was conducted. The assessment was carried out at four time points: (0 hpi, 12 hpi, 72 hpi, and 8 dpi) using qRT-PCR (**Figure 4.5, right hand side**). In general, the results showed a significant decrease in gene expression of all four tested candidate genes in infected seedlings at 72 hpi and 8 dpi, as compared to the FoMV: EV. This suggests that the four candidate gene constructs were effective in inducing gene silencing in the infected seedlings. FoMV:PK_Tyr_Ser-Thr/Pkinase showed significant reduction in expression at 72 hpi in NIL2 compared to the empty vector (**Figure 4.5A2**). Additionally, there was a significant reduction in expression of this gene in all three genotypes 8 dpi. However, no significant reduction in expression at 0 and 12 hpi was detected for all three genotypes. Moreover, FoMV:LTP_2/Tryp_alpha_amyl showed a significant reduction in expression levels at 72 hpi in NIL2 and TIL11, as well as at 8 dpi in all three genotypes (**Figure 4.5B2**). However, this gene was not silenced at 72 hpi in NIL1 and TIL11. Similarly, FoMV: TPR_1 was detected at 12 hours post *U. maydis* inoculation in NIL2, and at 72 hpi and 8 dpi in

all three genotypes (**Figure 4.5C2**). However, FoMV:TPR_1 did not exhibit a significant reduction at 0 hpi for all genotypes and 12 hpi in NIL1 and TIL11. However, no significant reduction was observed at 0 hpi and 12 hpi for all genotypes and at 72 hpi in NIL1. Regarding FoMV:No Pfam, a significant reduction in expression levels was observed at 72 hpi in NIL2 and 8 dpi in all three genotypes compared to FoMV:EV (**Figure 4.5D2**). However, there was no significant reduction at 0 and 12 hpi for all genotypes and 72 hpi in NIL1 and TIL11.

The observed difference in gene expression at different time points post-inoculation in TIL11 and the two NILs may be due to the timing of the plant's response to the pathogen *U. maydis*. All four genes showed a significant reduction in expression at 72 hpi and 8 dpi, suggesting that the plant's defense mechanisms may take some time to recognize and respond to the pathogen. Therefore, there was no significant reduction or silencing of gene expression at 0 hpi and 12 hpi. However, at 72 hpi and 8 dpi, the plant may have fully recognized the pathogen and initiated a stronger defense response, leading to the observed gene silencing. Moreover, the effectiveness of the plant's defense mechanisms may change over time, resulting in differences in gene expression levels at different time points.

To evaluate the combined effects of each construct, we computed the overall relative expression levels by aggregating data from all time points and comparing the gene expression relative to an empty vector and mock inoculation (**Figure 4.5E**). **Table 4.2** presents the reduction in relative expression for all four FoMV constructs in TIL11, NIL1, and NIL2 compared to FoMV: EV. Among these, the most significant reduction in relative expression was observed in TIL11 with FoMV:LTP_2/Tryp_alpha_amyl, while the smallest reduction was observed with FoMV:No Pfam when compared to their respective empty vectors. In the case of NIL1, the highest reduction in relative expression level was observed with FoMV: TPR_1,

whereas PK_Tyr_Ser_Thr/Pkinase and LTP_2 exhibited the highest decline in expression in NIL2. These findings indicate that VIGS effectively silenced the defense genes, rendering the plants more susceptible to *U. maydis.*

DISCUSSION

Although several studies have confirmed that FoMV has the ability to infect maize lines (Mei et al. 2016; Mei et al. 2019; Bouton et al. 2018), this is the first study of FoMV based gene silencing carried out in teosinte and the two NILs. qRT-PCR analysis revealed that all three genotypes did not significantly reduce gene expression at 0 hpi. This is likely because the pathogen did not have sufficient time to impact gene expression at this early time point. Plant defense responses typically take several hours to a few days to fully induce plant defenses after pathogen infection. Therefore, gene expression levels in all three genotypes may remain stable until later time points post-inoculation.

Out of the four defense-associated genes, only FoMV: TPR_1 showed a significant decrease in expression at 12 hpi in NIL2, while it remained unchanged in TIL11 and NIL1. FoMV:PK_Tyr_Ser-Thr/Pkinase and FoMV:No Pfam also exhibited a significant reduction in expression at 72 hpi in NIL2, but not in the other two genotypes. FoMV:LTP_2/Tryp_alpha_amyl showed a significant decrease in expression at 72 hpi in NIL2 and TIL11, but not in NIL1. The variation in gene expression may be due to genetic differences that affect gene expression or regulation. During defense responses, different genes are involved at different stages, which are coordinated by signaling pathways. The genes that were not expressed in some genotypes at 12 hpi and 72 hpi may still play a role in the later stages of the defense responses. Further research is necessary to understand the temporal dynamics of gene expression in response to the pathogen and the underlying mechanisms involved.

We observed a significant decrease in the expression of FoMV:TPR_1 at 72 hpi in all three genotypes. Additionally, we detected a significant reduction in the expression of all four genes at 8 dpi in all three genotypes. These findings suggest that the host's defense response was weakened, possibly due to the silencing of these defense associated genes. Therefore, these genes could be crucial in mounting an effective defense response against *U. maydis*.

In order to use the FoMV-VIGS based system, we first evaluated its ability to infect TIL11 and the NILs. Rub inoculation, agro-inoculation, and vascular puncture inoculation are commonly used methods for VIGS research. However, the efficiency of virus inoculation varies depending on the specific virus and inoculation method used. For instance, CMV infection in maize is best achieved through vascular puncture inoculation of maize kernels, while the rub inoculation with CMV results in a low percentage of infected plants (Wang et al. 2016). BMV infection in maize seedlings, on the other hand, can be accomplished via rub inoculation (van der Linde et al. 2011). FoMV-infected maize seedlings have been shown to have high infection efficiency when inoculated via agro-inoculation methods (Beernink et al. 2021). We conducted a preliminary experiment to compare the rub inoculation, agro-inoculation, and vascular-puncture inoculation methods. Agro-inoculation was a more efficient and less time-consuming approach to induce FoMV infection. Therefore, for our current study, we chose the agro-inoculation method to deliver FoMV with a gene of interest and an empty vector. This allowed us to achieve successful gene silencing in TIL11 and both NILs.

Research has shown that the specific construct used impacts the efficiency of VIGS. Insert stability of plant viral vectors largely depends on the insert size and sequence, as highlighted by several studies (Yamagishi et al. 2015). For instance, the Potato virus X vectors have been observed to experience a partial or complete loss of inserted sequences, especially

when the insert size is large (Avesani et al. 2007; Dickmeis et al. 2014). Similarly, Mei et al. 2016 observed similar issues linked to insert size and leaf stage sampled. Successful VIGS typically require small sequence inserts, not exceeding 400 bp, yet instability can still be challenging even for these constructs (Bruun-Rasmussen et al. 2007; Mei et al. 2016). Therefore, in our study, we designed constructs with a range of 60-85 bp in length and inoculated young seedlings aged 4-6 days to minimize instability. We also ensured that environmental variables did not affect the inoculation efficiencies by conducting experiments in the same facility for both NILs and TIL11.

In the agro-inoculation method, the plant, virus, and *Agrobacterium* strain must coordinate for successful FoMV infection. The *Agrobacterium* strain must be capable of infecting cells within the plant tissue to deliver the T-DNA carrying the viral genome, while the plant must also be susceptible to the virus to initiate viral replication and systemic infection. Additionally, the injection location plays a critical role in the success of the agro-inoculation procedure (Zaidi and Mansoor 2017; Scholthof et al. 1996). To ensure effective FoMV infection, we performed the agro-inoculation method, ensuring that the inoculum was delivered precisely to the meristem location and carefully filling the leaf whorl. Additionally, accurate evaluation of viral infection relies heavily on the proper selection of leaf samples and the timing of their collection for qRT-PCR analysis (Met et al. 2016; Beernink et al. 2021). Our study identified leaf numbers by starting with the first thumb leaf and counting upwards, specifically focusing on the fourth to sixth leaves since these exhibited FoMV symptoms during this developmental stage.

In summary, this study shows that VIGS can be used for functional genomics studies in both teosinte plants and NILs. The study found a significant reduction in gene expression for four genes in TIL11 and two NILs. This suggests that these genes play a functional role in

pathogen defense, which was demonstrated by an increased susceptibility to *U. maydis* infection when the genes were downregulated in comparison to the control. Since all four genes were individually silenced, the overall percentage reduction in gene expression for each gene was not very high. In addition, observation of disease symptoms suggests that each gene has a role in the plant's defense mechanism against the specific disease. Resistance to *U. maydis* is quantitative, therefore it is likely that each of the four genes contributes to resistance gradaully. This supports the idea that the plant's defense mechanisms are a complex, multi-gene system, where each gene contributes incrementally to the overall defense response. To summarize, all four genes are necessary for an effective defense response, but none of them alone can provide complete resistance to the disease. VIGS is a transient assay, so the silencing effect is unstable over long periods. To confirm the observed phenotypes, further validation of these four genes using more stable assays such as CRISPR/Cas9-mediated gene editing is essential to determine the definitive evidence of gene function.

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Table 4.1. Four candidate genes identified in the 3.9 Mbp region of chromosome 9 used for virus induced gene silencing

	Protein Family Domain	Full Name	Function
1.	PK_Tyr_Ser-Thr/ Pkinase	Protein kinase tyrosine serine threonine/protein kinase domain	Protein kinase tyrosine serine threonine is a protein kinase domain that is involved in the phosphorylation of tyrosine, serine, and threonine residues in protein substrates.
2.	LTP_2;Tryp_alpha_amyl	Lipid transfer protein_2 family/ Trypsin-like serine protease domain alpha amylase inhibitor	Lipid transfer protein family is involved in the transfer of lipids between membranes in cells. They have been shown to play a role in various cellular processes, including signal transduction, stress response, inhibit fungal and bacterial growth, regulating cell wall structure, phytohormone signaling, and defense against pathogens.
3.	TPR_1	Tetratricopeptide repeat domain 1	Tetratricopeptide repeat (TPR) domain 1 containing proteins in plants are involved in protein-protein interactions.
4.	No Pfam		This gene does not have a protein family domain.

Table 4.2. Relative expression reduction of four constructs in TIL11, NIL1, and NIL2 after inoculation using qRT-PCR and pooling all time points (0, 12 and 72 hours post inoculation and 8 days post inoculation).

Genes	Genotypes	Relative expression reduction %
FoMV:LTP_2	NIL1	19.14
	NIL2	30.82
	TIL11 (*Zea mays* ssp. *parviglumis*)	38.17
FoMV:TPR_1	NIL1	31.08
	NIL2	29.37
	TIL11 (*Zea mays* ssp. *parviglumis*)	24.88
FoMV:No Pfam	NIL1	18.30
	NIL2	29.55
	TIL11 (*Zea mays* ssp. *parviglumis*)	15.34
FoMV:PK_Tyr_Ser-Thr	NIL1	20.28
	NIL2	30.54
	TIL11 *(Zea mays ssp. parviglumis)*	30.40

FoMV represents *Foxtail mosaic virus*, PK_Tyr_Ser-Thr represents Protein kinase tyrosine serine threonine/protein kinase domain, LTP_2 represents lipid transfer protein_2 family/ Trypsin-like serine protease domain alpha amylase inhibitor, TPR_1 represents Tetratricopeptide repeat domain 1, No Pfam represents gene that does not have protein family domain.

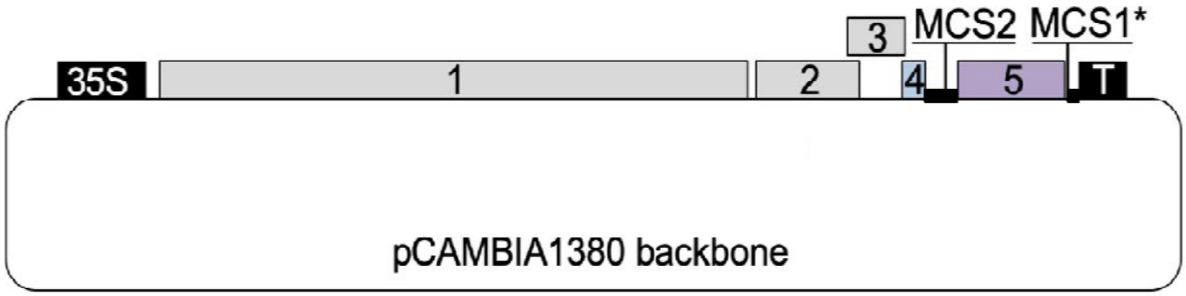
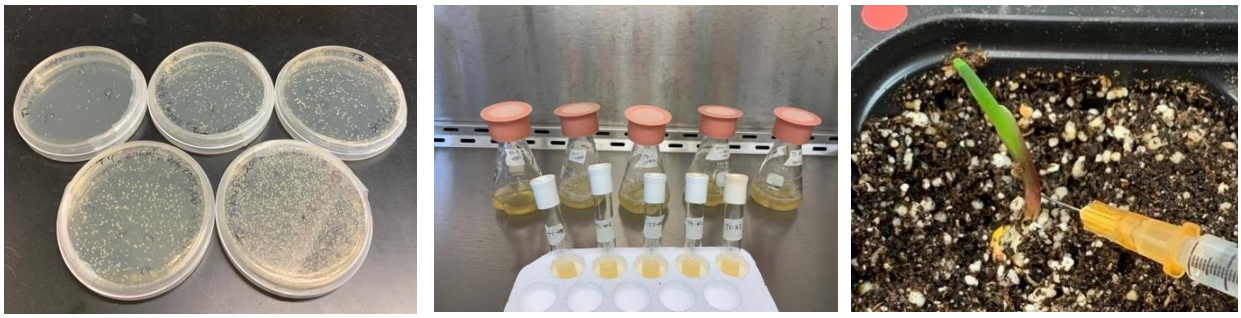


Figure 4.1. Graphical representation of the *Foxtail mosaic virus* (FoMV) T-DNA clone used for agro-inoculation in maize. FoMV vector contains two multiple cloning sites (MCS1 and MCS2). The empty vector FoMV:EV is 7, 269 bp and contains no inserts in either MCS site. Gene silencing using the FoMV vector can be achieved by inserting target gene fragments into the Multiple cloning site 1, typically in the antisense orientation.



A **B** **C**

Figure 4.2. Agro-inoculation of TIL11, NIL1 and NIL2 with *Foxtail mosaic virus* (FoMV): PK_Tyr_Ser-Thr, FoMV: LTP_2, FoMV: TPR_1, and FoMV: No Pfam, and FoMV: empty vector. **(A)** LB plates with the four constructs and empty vectors. **(B)** Agrobacterium cultures were prepared a day before agro-inoculation. **(C)** Agro-inoculation was performed in 4-6 days old TIL11, NIL1, and NIL2 seedlings.

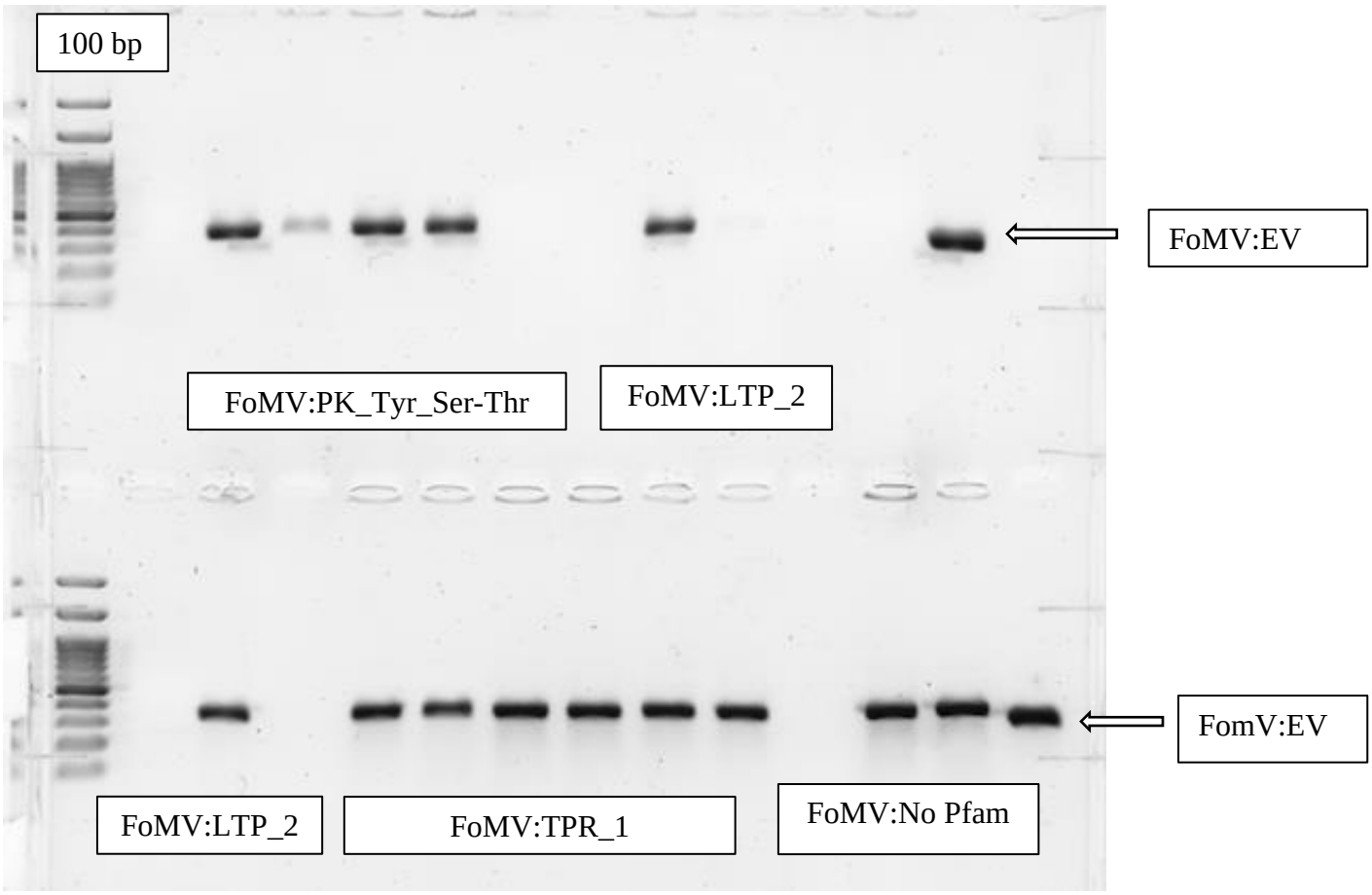
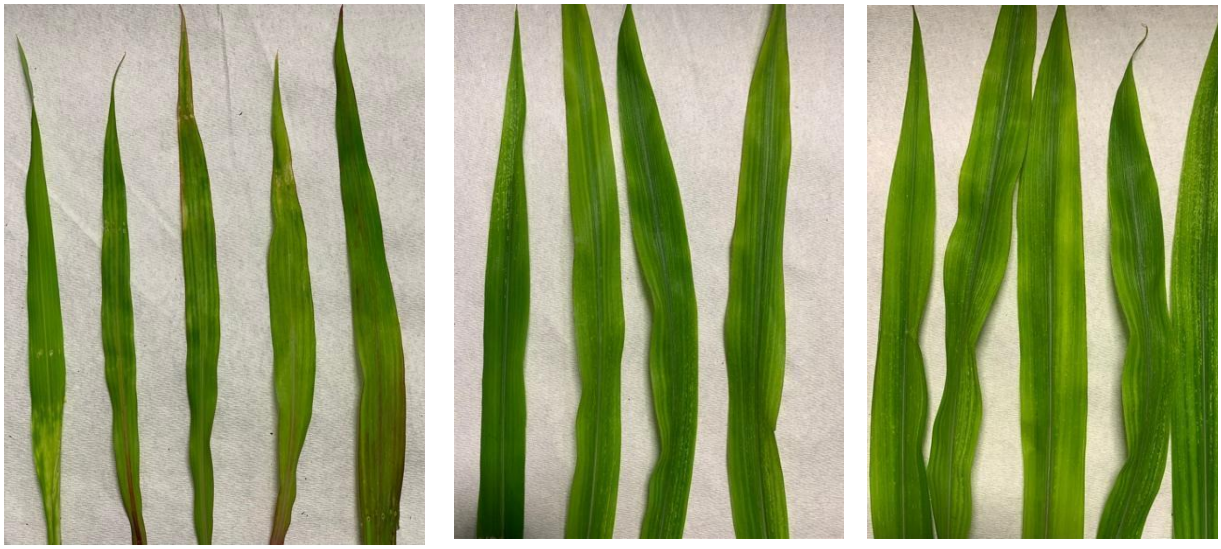


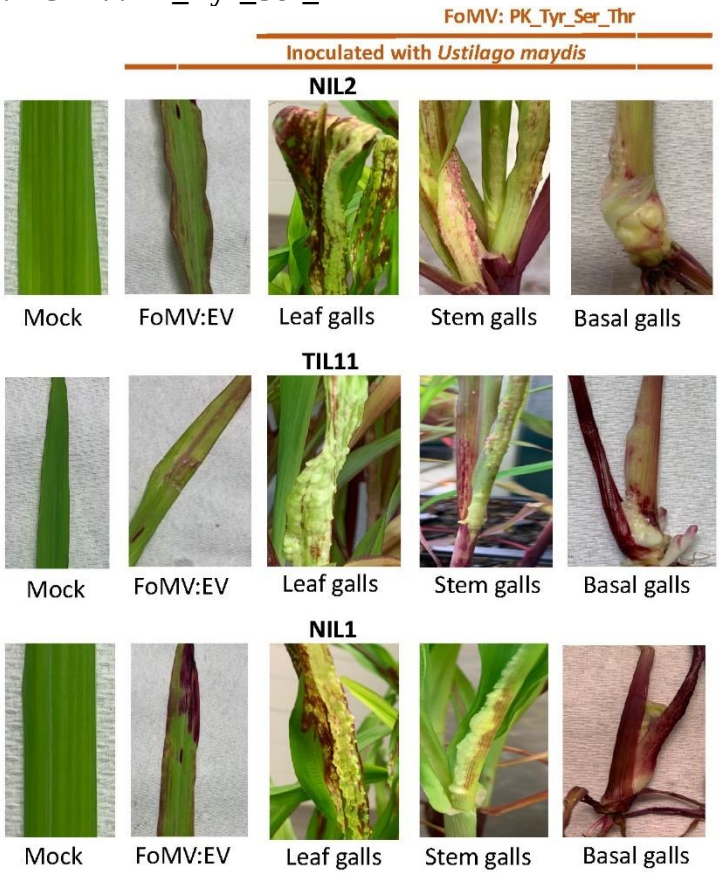
Figure 4.3. Successful transformation of four plasmid DNA carrying target genes. The transfer of five constructs, namely FoMV:PK_Tyr_Ser-Thr, FoMV:LTP_2, FoMV:TPR_1, FoMV:No Pfam, and FoMV:EV, into *Agrobacterium* strain GV-3101 was accomplished through gel electrophoresis. The PCR primers were utilized to generate an amplicon that covers MCS1. FoMV:EV served as a positive control with a length of 315 bp, whereas the four plasmid DNA-containing target genes had a length slightly above 315 bp (375-386), confirming their presence. The gel electrophoresis image revealed the presence of four bands on the left side of the first tier for FoMV:PK_Tyr_Ser-Thr, one single band on the right side and one single band on the left side of the second tier for FoMV:LTP_2. Six bands on the middle of the second tier indicated the presence of FoMV:TPR_1, while one single band on the right side and two bands on the left side of the second tier indicated the existence of FoMV:No Pfam.



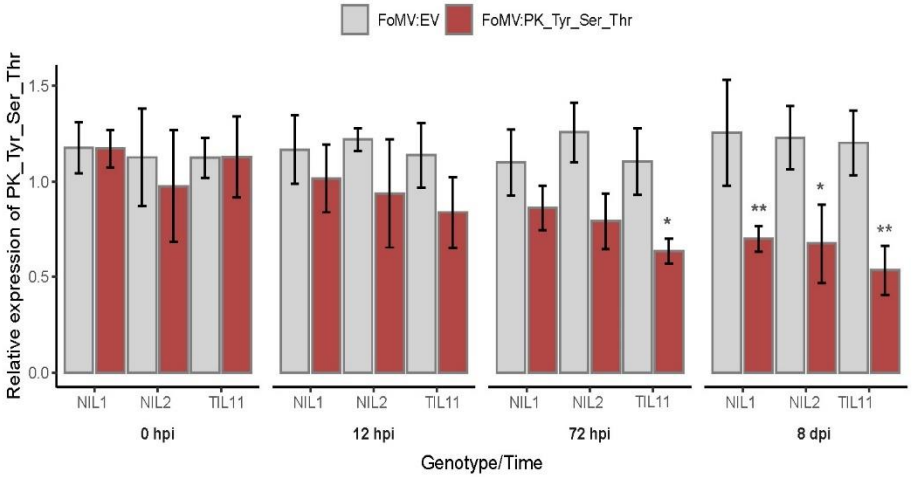
A. TIL 11 **B. NIL1** **C. NIL2**

Figure 4.4. Phenotypic evaluation of the *Foxtail mosaic virus* (FoMV) infection in TIL11, NIL1, and NIL2. Seedlings agroinjected with FoMV:PK_Tyr_Ser-Thr, FoMV:LTP_2, FoMV:TPR_1, and FoMV:No Pfam, and FoMV:EV developed mosaic symptoms in leaves of all three genotypes, TIL11 in **(A)**, NIL1 **(B)**, and NIL2 **(C)**.

A: FOMV:PK_Tyr_Ser_Thr

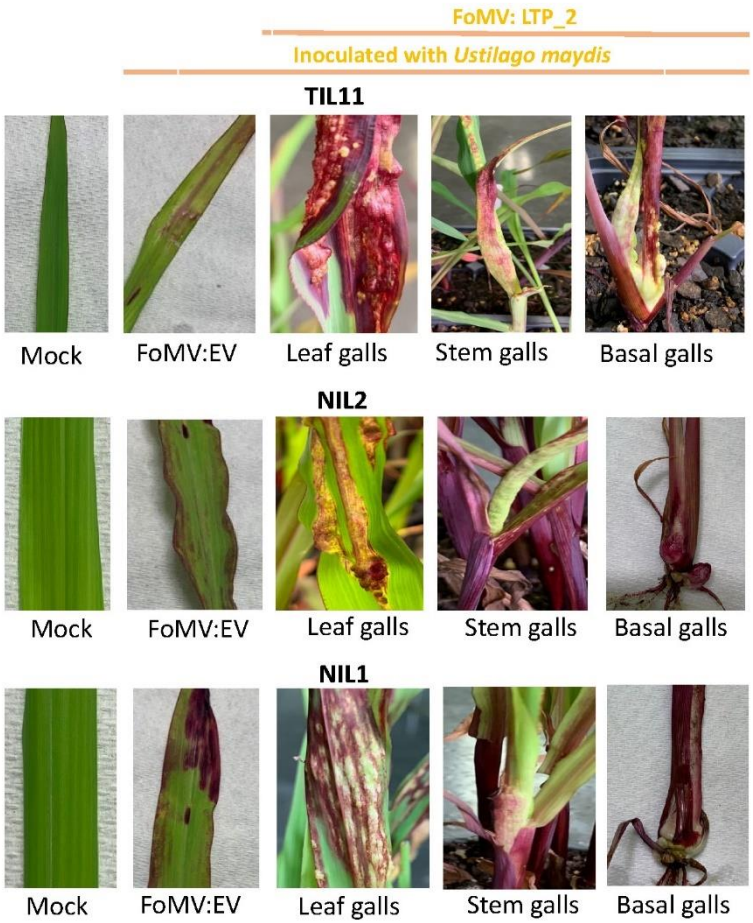


A1

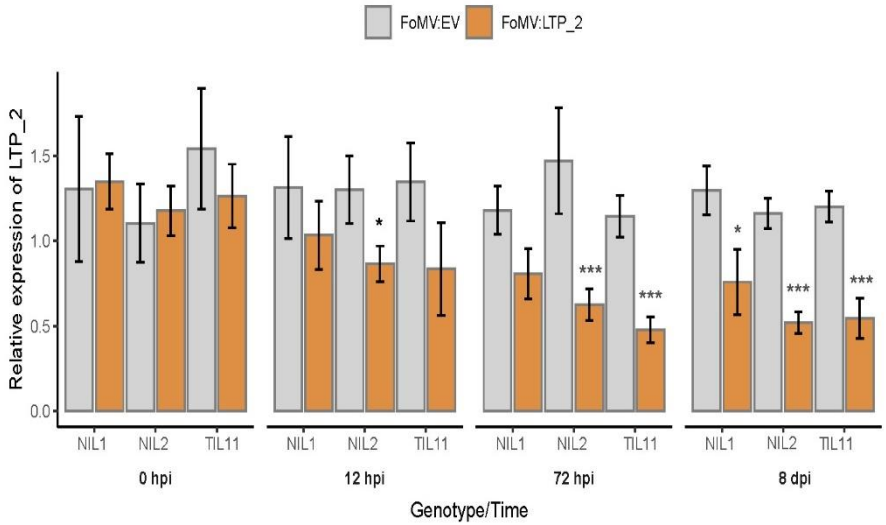


A2

B: FOMV:LTP_2

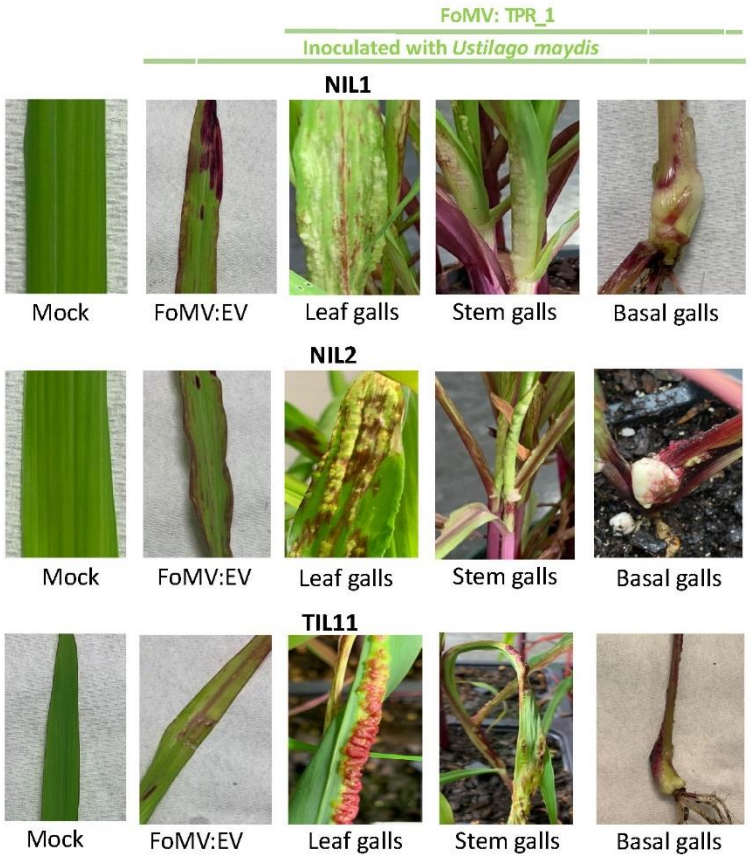


B1

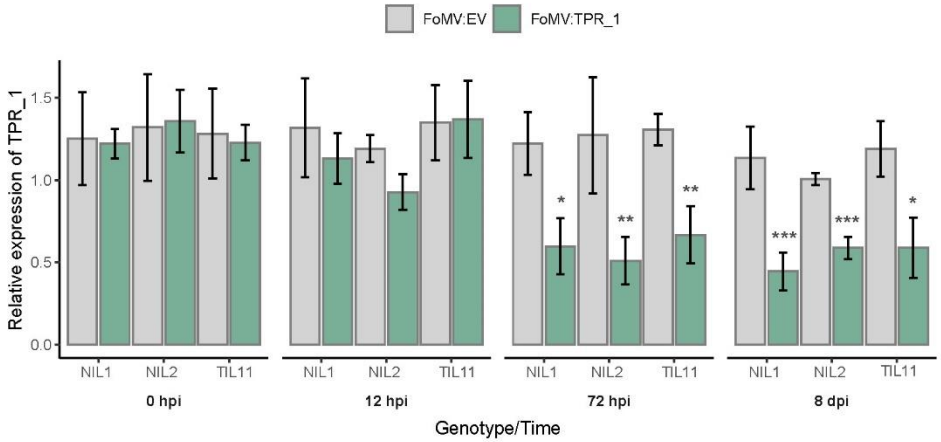


B2

C: FOMV:TPR_1

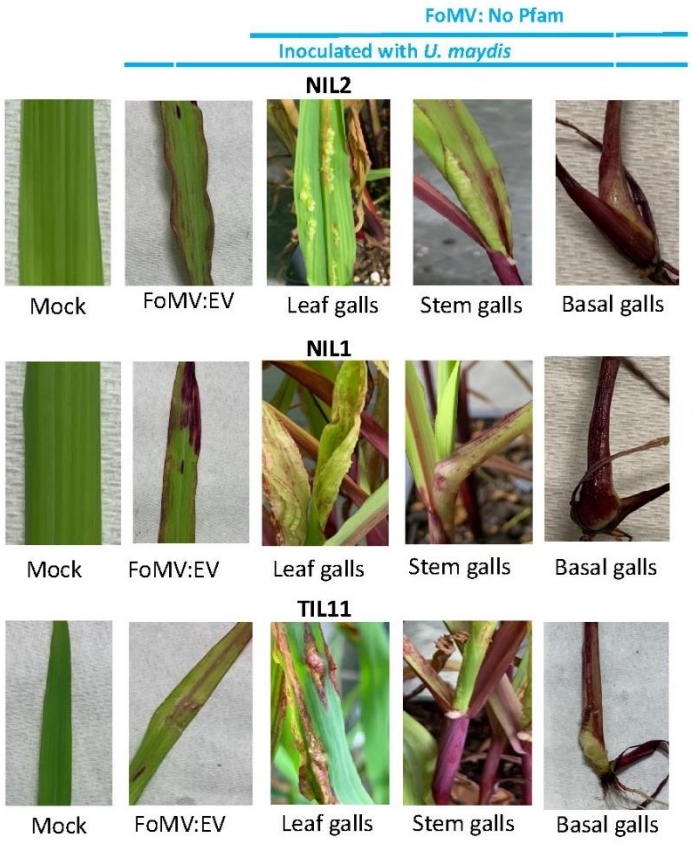


C1

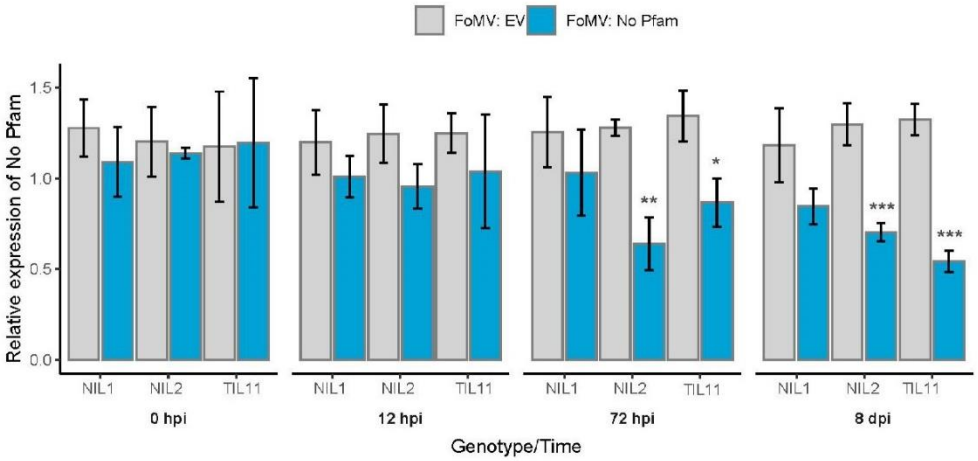


C2

D:FOMV:No Pfam



D1



D2

E: Overall effects

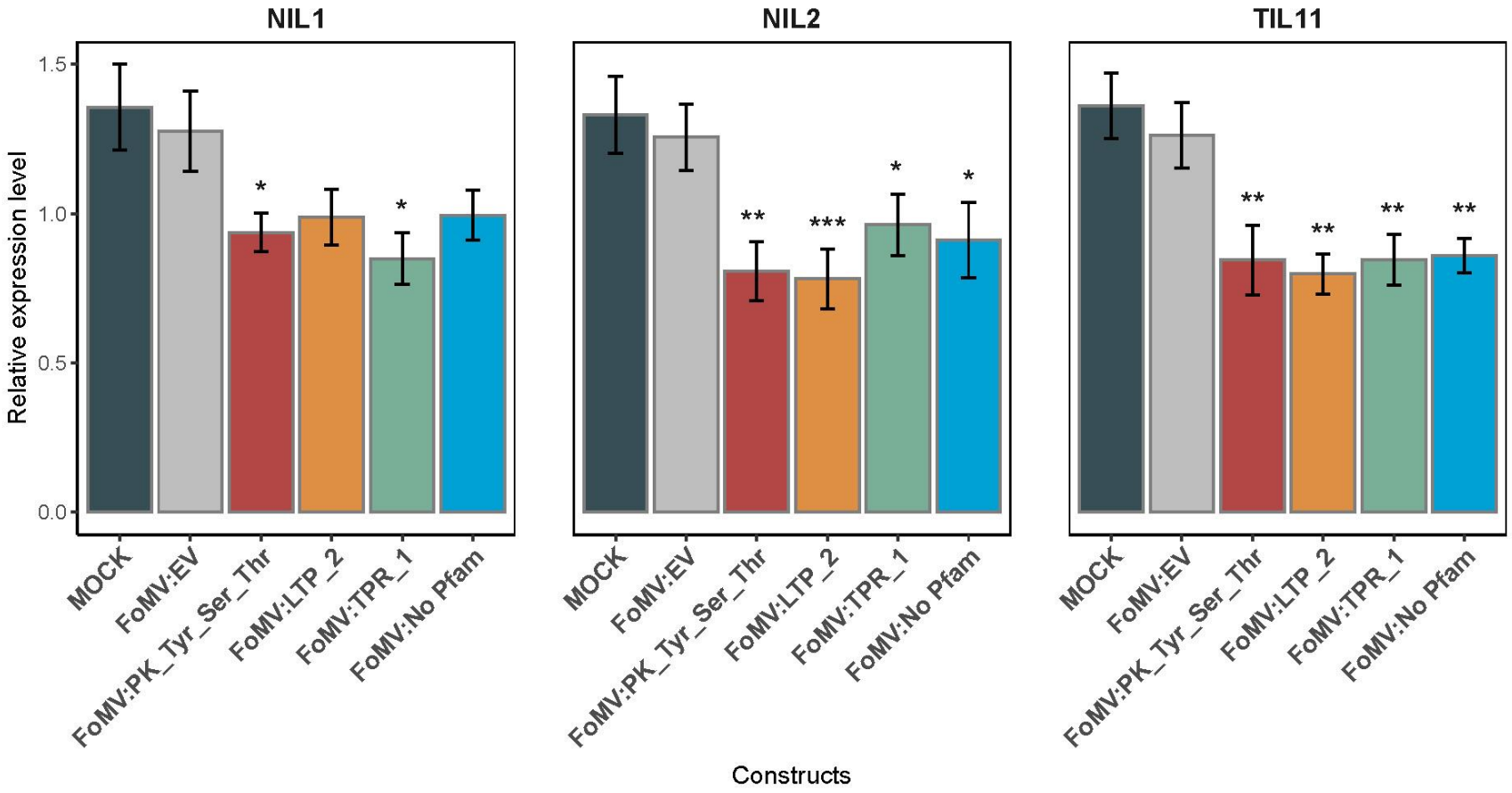


Figure 4.5: Phenotypic evaluation and relative expression of TIL11, NIL1, and NIL2 after agro-inoculation with mock, *Foxtail mosaic virus* (FoMV): empty vector, and FoMV gene silencing constructs using qRT-PCR. The phenotypic assessment was carried out in mock, FoMV:EV and FOMV constructs at 8 days post-inoculation (dpi) with *U. maydis* (left hand side). Plants agro-inoculated

with FoMV:EV and FoMV constructs were inoculated with *Ustilago maydis*. The bar graph represents the qRT-PCR relative expression values for each candidate gene at 0, 12, 72 hour post inoculation (hpi), and 8 dpi with *U. maydis* (righthand side). **A1** represents phenotype and **A2** is the relative expression of FoMV:PK_Tyr_Ser-Thr in TIL11, NIL1, and NIL2 at 0 hpi, 12 hrs, 72 hrs, and 8 days post-inoculation. **B1** represent phenotype and **B2** is **the** relative expression of FoMV:TPR_1 in TIL11, NIL1, and NIL2. **C1** represent phenotype and **C2** is the relative expression of LTP_2 in TIL11, NIL1, and NIL2. **D1** represent phenotype and D2 is the relative expression of No Pfam in TIL11, NIL1, and NIL2. **E** shows combined relative expression levels by pooling data from all time points for mock, FoMV:EV and each FoMV construct ($p \leqq 0.05$ *; $p \leqq 0.01$ **; $p \leqq 0.001$***; Student's t-test; Mean values $\pm$ standard deviation (sd) from three technical replicates are shown). For the phenotypic assessment, a disease rating scale of (0-5) was used to assess the disease severity at 8 dpi. A resistance reaction comprised of scale of 0-2, where 0- completely healthy, 1C- chlorosis, 1A- anthocyanin, 2- minor leaf galls, whereas a susceptible reaction comprised of a rating scale from 3-5, where 3- major leaf and stem galls, 4- basal galls, and 5- death of the plant.

CHAPTER 5

CONCLUSION

Various strategies are available to manage corn smut, such as crop rotation, sanitation, seed treatments, and foliar fungicide application, but no maize cultivars are entirely resistant to *Ustilago maydis*. Wild relatives of maize have demonstrated high levels of resistance to pests and diseases, making host resistance likely the most practical and cost effective method for managing corn smut in regions where *U. maydis* is prevalent. Despite the potential for research to enhance resistance in domesticated maize, investigations into wild-relative introgressions for disease resistance against *U. maydis* have been limited. Therefore, the primary objective of this study was to identify new sources of resistance against *U. maydis* from the wild ancestor teosinte (*Z. mays* ssp. *parviglumis*/teosinte inbred line-TIL11). In previous research, 100 NILs were generated by crossing a wild teosinte ancestor with domesticated maize. The phenotypic and genotypic evaluations revealed a 3.9 Mbp teosinte introgressed region in the two NILs that contributed to the resistance phenotype in two NILs but was absent in the remaining 98 NILs. Therefore, the primary goal of this research was to identify candidate genes in the 3.9 Mbp-teosinte introgressed region contributing to the resistance observed in the two NILs and TIL11. This research consisted of three different studies in order to achieve this goal.

In Chapter 2, we conducted a comparative genome analysis of the 3.9 Mbp region between the resistant parent (TIL11) and the susceptible parent (B73) to identify new sources of resistance to *U. maydis*. Our analysis identified a total of 24 candidate genes that encode Pfam

domains associated with disease resistance, pathogenesis related, and defense genes. These findings suggest that resistance to *U. maydis* is a complex trait influenced by multiple genes with unique and shared roles in the defense mechanism. Our comparative analysis of domesticated maize and the wild ancestor of maize-teosinte revealed that some of the genes present in teosinte had been lost in modern maize, making it more susceptible to *U. maydis*. However, some genes have been conserved in B73 due to their gain during domestication, leading to variations in resistance levels between wild ancestors and domesticated maize. The identification of unique resistance genes in the wild ancestor TIL11 suggests additional genetic variation between TIL11 and domesticated maize. Furthermore, identification of candidate genes in both TIL11 and B73-NAM lines indicates the need for further investigations into the genetic basis of resistance to *U. maydis*. Exploiting genes unique to TIL11 could lead to the development of new maize varieties with improved resistance to *U. maydis* and other pathogens.

In Chapter 3, RNA-seq analysis was conducted on TIL11, B73, and the two NILs to identify DEGs in response to *U. maydis*. By analyzing the 3.9 Mbp region, we found 24 DEGs, with only four upregulated and significantly expressed in TIL11 and the two NILs. We further validated these genes through qRT-PCR, and the results were consistent with the RNA-Seq data. It is worth noting that a Peptidase_S10 unique to TIL11 did not show expression in the RNA-Seq data, but it is unclear whether it is non-functional or regulated differently in teosinte compared to B73. We speculate that the experimental conditions may not have induced its expression, or its expression levels were too low to be detected. We also found specific candidate genes with Pfam domains associated with resistance and defense functions expressed in both TIL11 and B73, as well as in the NILs, but at significantly higher levels in teosinte and the two NILs. These results suggest that these genes may play crucial roles in the plant disease resistance and defense

response to *U. maydis* in TIL11 and the two NILs. These results provide insights into the genetic basis of resistance to *U. maydis* in teosinte and identify potential candidate genes for improving resistance in domesticated maize.

In Chapter 4, we used VIGS to characterize and verify the resistance function of the four genes that were expressed in response to *U. maydis* inoculation. In most cases, the silencing of these four genes occurred at 72 hours post inoculation (hpi) and 8 days post-inoculation (dpi) in two NILs and TIL11, but some weaker silencing also occurred 12 hpi. The genes silenced at 12 hours post-inoculation may be crucial for the early stages of pathogen recognition and initiation of defense signaling pathways. In contrast, those silenced at 72 hpi may be more critical for downstream defense responses. Genes silenced at the 8 dpi may be involved in long-term defense responses or maintaining plant resistance to the pathogen. Therefore, each gene has a specific and unique role in the plant's defense response, and their coordinated expression is essential for effective defenses. Exploring the distinct functions of these genes and their interplay with other genes and pathways can provide significant insights into the mechanisms governing the response to infection. For future work, we propose silencing the four candidate genes in different combinations to see more of an effect, as resistance to *U. maydis* is quantitative. We also propose leveraging CRISPR/Cas9 technology to generate knockout plants lacking the targeted genes and examine their response to the pathogen, which can further verify the function of the silenced genes.

Overall, this study supports the hypothesis that a wild ancestor of maize may possess a higher level of resistance to *U. maydis* than domesticated maize varieties. These findings could guide further research on the genetic basis of resistance to *U. maydis* and aid in developing new maize varieties with improved resistance. Moreover, since ancient resistance genes from wild

relatives are more likely to provide durable resistance, this work could have significant implications for characterizing a resistance mechanism that may be more durable and developing novel strategies to control *U. maydis* by dissecting plant-pathogen interactions.

APPENDICES

APPENDIX A

EFFECTS OF POLLINATION ON *USTILAGO MAYDIS* DISEASE DEVELOPMENT ON THE EARS OF MAIZE AND NEAR-ISOGENIC LINES

ABSTRACT

Ustilago maydis, the causal agent of corn smut, results in significant economic loss in current maize cultivars. A key characteristic of successful plant pathogens is their innate ability to utilize the intimate plant-pathogen relationship to influence disease development. The plant genotype and successful transmission of the pathogen are also important factors that influence disease development. Therefore, these same factors may also influence the successful transmission of fungal spores. A greenhouse experiment was conducted to evaluate the effects of pollination on maize ears inculated with *U. maydis*. Four susceptible maize genotypes (B73, H95, Mo17, and Golden Bantam) and two resistant maize-teosinte near-isogenic lines (NILs) (MT-NIL1 and MT-NIL2) were evaluated. A total of 360 plants were inoculated with *U. maydis* through the silk channel. Controlled crosses were made with hand-pollinated and unpollinated plants after silk emergence. When compared to unpollinated plants, ears of all hand-pollinated plants had significantly lower disease incidence, gall number, gall weight, area under disease progress curve (AUDPC) and disease severity. While the magnitude of the effecs of pollination on diseae development varied among plant genotypes, the two hand-pollinated resistant NILs demonstrated significantly less disease development than the unpollinated resistant NILs, as well as all of the susceptible maize genotypes. The findings from this study demonstrated pollination reduces *U. maydis* infection, with the effects being more prominent in pollinated resistant genotypes with an introgressed region from teosinte, suggesting pollination has an influence on *U. maydis* infection and host plant resistance and genes from teosinte are effective against *U. maydis*.

Keywords: Corn smut, Maize, Near-isogenic lines, Disease incidence, Disease severity**,** Pollination

INTRODUCTION

Corn smut disease, caused by a member of the fungal phylum basidiomycota, *Ustilago maydis* is a major problem in maize production worldwide. In spite of several control measures, corn smut causes a significant economic loss of approximately $1 billion annually in the United States in maize inbred lines (Smith 2011). Mueller et al. (2020) reported 0.3 million bushels of corn yield loss in twenty-eight U.S. states and 1.8 million bushels yield loss in Ontario, Canada in 2021. In addition, smutted ears render corn inedible and unmarketable and create additional costs during harvesting and processing (du Toit and Pataky 1999b).

While *U. maydis* infects many plant organs such as leaf, stem, tassels, and ears, infections of the ears are most common (Basse and Steinberg 2004; Kamper et al. 2006). Maize ears are infected by *U. maydis* primarily through silks. The silks extend from the ovaries and emerge through a silk channel formed by husk leaves at the tip of the ear. In a study by Snetselaar and Mims (1993), infection of maize silks by *U. maydis* was documented with micrographs where silks were inoculated with different aqueous suspensions of smut sporidia. When stigmas were inoculated with sporidia that had compatible alleles at both mating loci (a and b), sporidia mated in pairs using a conjugation tube on the silk surface, and each pair formed a dikaryotic infection hypha that grew rapidly across the stigma surface and penetrated the silk by means of an appressorium. In natural infections, *U. maydis* sporidia are disseminated by wind or rain and are deposited onto newly emerged silks. The dikaryotic phase of the corn smut pathogen grows

down the silk and into the developing ovary to form galls (Snetselaar and Mims 1993; Pataky et al. 1995).

Several studies describing corn smut management for sweet corn have suggested pollination as an important factor that affects the susceptibility of corn ears to *U. maydis* infection *(*du Toit and Pataky 1999a*)*. Snetselaar and Mims (1993) hypothesized that ears remain susceptible to infection by *U. maydis* for a longer time when silks are not pollinated, than when silks are pollinated. Past studies have reported mechanisms such as the formation of an abscission zone at the base of the silk after successful pollination (Heslop-Harrison et al. 1984; Snetselaar et al. 2001), potentially precluding access to the fertilized ovary by pathogens that infect kernels via the silk. Studies have found maize kernels appear to be protected from infection by *U. maydis* after the formation of the abscission zone because *U. maydis* infection filaments are unable to grow past this layer of dead cells (Snetselaar and Mims 1993; Snetselaar et al. 2001).

Although several practices have been developed to manage *U. maydis*, resistance is quantitatively inherited lending to the difficulty of managing the disease (Ruan et al. 2021). The use of some genetic introductions from wild relatives has emerged as a sustainable and effective method to improve the resistance of crop plants to several important pathogens (Hajjar and Hodgkin 2007). For example, teosinte, a wild ancestor of maize, confered resistance to several pathogens, including wheat rust and anthracnose of bean and can be used for the development of resistant maize cultivars (de Lange et al. 2014). In this study, four susceptible maize genotypes (B73, H95, Mo17, and Golden Bantam) and two resistant maize-teosinte near-isogenic lines (NILs) (MT-NIL1=NIL1 and MT-NIL2=NIL2 as described in Chapters 1 through 5) were evaluated for resistance to *U. maydis* and the effect of pollination on resistance at the adult stage.

The two resistant NILs were identified in a maize-teosinte introgression population created by Dr. Sherry Flint-Garcia at the University of Missouri, USDA-ARS (Chavan and Smith 2014). The population was created by backcrossing ten different teosinte *Zea mays* ssp. *parviglumis* accessions into the maize inbred B73 background, producing 900 teosinte introgression lines. A "minimum tilling path" of 100 lines that contained almost the entire teosinte genome, with each introgression line carrying a single different region from the teosinte genome, was created. Out of the 100 teosinte introgression lines, two maize-teosinte NILs (MT-NIL1; MT-NIL2) were resistant to *U. maydis* at the seedling stage and were found to carry a 3.9 Mbp teosinte introgressed region on the short arm of chromosome 9. This indicated that the 3.9 Mbp teosinte introgressed region contained the genes associated with the resistance phenotype.

Previous studies, particularly with sweet corn, demonstrated pollination decreased *U. maydis* disease incidence and severity (du Toit and Pataky 1999a; Pataky and Chandler 2003), while Ngugi et al. (2002) found that early pollination showed a reduction in hyphal growth of *Monilinia vaccinii-corymbosi* and fruit disease incidence in blueberries. However, none of these studies explored the effects of pollination on genotypes that are resistant to *U. maydis*. The objective of this study was twofold: 1) examine the resistance of *U. maydis* at the adult stage (ears and tassels), and 2) assess the impact of pollination on two resistant maize-teosinte NILs and four susceptible maize genotypes to determine if pollination is a governing factor for disease development with genes from teosinte wild progenitor. We hypothesize that the two hand-pollinated maize teosinte-introgressed NILs are more resistant to *U. maydis* than the unpollinated NILs and four maize genotypes due to two factors: 1) the pollinated maize-teosinte NILs maintain the genes that are associated with resistance to *U. maydis*, and 2) pollination has a negative impact on disease development. To test this hypothesis, the phenotypic effects of *U.*

maydis on ear gall development of pollinated and unpollinated maize-teosinte NILs (MT-NIL1 and MT-NIL2) and four susceptible maize genotypes (B73, H95, Golden Bantam, and Mo17) were compared.

MATERIALS AND METHODS

Plant Materials and Experimental Design

Four maize genotypes (B73, H95, Mo17, and Golden Bantam) obtained from the USDA National Plant Germplasm System, USA were used for this study. Two maize-teosinte NILs (MT-NIL1; MT-NIL2) resistant to *U. maydis* identified in a population obtained from Dr. Sherry Flint-Garcia at the University of Missouri were also used in this study. Experiments were conducted in the summer of 2020 and 2021 in the South Milledge Greenhouse located at the University of Georgia, Athens, Georgia. Experiments were conducted on hand-pollinated and unpollinated ears inoculated with *U. maydis* using a completely randomized block two factorial design with three replicates for each genotype.

Pollination experiment plants were planted and maintained in a greenhouse separate from unpollinated (controlled) experiment plants to prevent unwanted pollination. Ears were not covered on plants used in the pollination experiment. Tassel bags were used to collect pollen for hand-pollinations. Hand-pollinations were performed for the pollination experiment by self-pollinating each plant with the pollen collected in the tassel bag to insure pollination. Coversely, plant ear in the unpollinated experiment, were covered before silk emergence and remained covered through the entire experiment to prevent unwanted pollination. The experimental unit was a single plant grown in 10-inch-tall pots with a 10-inch diameter that contained a mixture of soil (SunGro Horticulture, professional growing mix). Osmocote Plus smart release plant food

was applied after seedling emergence and at the adult plant stage. Plants were watered twice a day and grown in greenhouses with day and night temperatures of 78°F and 72°F and day and night lengths of 16 hrs and 8 hrs, respectively. Plants with similar maturity were marked with tags in each replication.

Ustilago maydis Inoculum Preparation and Inoculation

Pollinated and unpollinated plant ears were inoculated with *U. maydis* wild-type mating strains (1/2: a1b1 and 2/9: a2b2). To prepare the inoculum, a sterile loop was used to streak each *U. maydis* strain onto a separate potato dextrose agar (PDA) plate. Streaked PDA plates were then placed into a 30°C incubator for two days. Growth of the pathogen cultures was monitored over a two-day period to ensure the cultures were growing well. A single colony for each strain was selected from PDA plates with a sterile toothpick and placed into a 3 ml potato dextrose broth (PDB). The 3 ml PDB culture for each strain was placed into a 30°C shaker for two days at 200 rpm. Growth of the culture was monitored for two days until the culture appeared milky white. Liquid cultures were removed from the shaker, and the concentration was measured at OD_{600} to ensure the cells were at an OD of 1.0 (~1 x 10^7 cells/ml) (Chavan and Smith 2014; Allen et al. 2011). *U. maydis* cell suspension cultures were brought to a final concentration of 1 x 10^6 cells/ml, using water in a final 30 ml volume. Equal volumes (15 ml each) of the two *U. maydis* strain cultures were combined in a single tube prior to inoculation to prepare a final 30 ml *U. maydis* sporidial suspension for inoculations.

Pollinated and unpollinated ears were inoculated by injecting *U. maydis* sporidial suspension into the silk channel 4 to 5 days after silk emergence when the silk was 1 to 2 cm long at the mid-silk growth stage (du Toit and Pataky 1999b). Each ear was needle inoculated

using a Covidien Monoject 3 ml syringe (REF 8881513934) equipped with an 18 G × 1 ½ inch hypodermic sterile needle.

Each *U. maydis* inoculation treatment consisted of 10 plants in each replication, for a total of 360 plants in both pollinated and unpollinated experiments (6 genotypes x 2 categories; pollinated and unpollinated x 10 inoculated ears x 3 replicates= 360 plants).

Disease Assessment and Statistical Analysis

Disease assessment for ear infection after inoculation with *U. maydis* was carried out at 7, 10, 14, and 21 days post-inoculation (DPI). Five phenotypic variables were evaluated: 1) disease incidence, 2) gall number, 3) gall weight, 4) disease severity, and 5) area under disease progress curve (AUDPC). Disease assessments were performed by a single person to avoid interrater error (Bock et al. 2008). A binary disease assessment approach was used to evaluate disease incidence (Weiland et al. 2018). A value of 1 was assigned when an ear developed galls and a 0 value was assigned otherwise. The average disease incidence of each inoculated plant was evaluated at 7, 10, 14, and 21 DPI. Data on gall number, gall weight in grams and severity were taken at 21 DPI. Disease severity of the ear was rated as the percentage of the pollinated or unpollinated ears covered with galls. Disease severity of ear galls was rated on a 0-5 disease rating scale (**Figure 1**), where 0 = uninfected ears, 1 =1-20% infected kernels, 2 = 21-40% infected ears, 3 = 41-60% infected ears, 4 = 61-80% infected ears, 5 = 81-100% representing death of the ears (Pataky 1991; Pataky and Chandler 2003; Pataky and Snetselaar 2006; Schilling et al. 2014; Redkar and Doehlemann 2016). The midpoint of each rating interval was utilized as a measure of disease severity for the analysis. AUDPC was calculated based on disease severity using the following equation (Simko and Piepho 2012),

$$AUDPC_i = \sum_{i=1}^{n-1} \left(\frac{y_i + y_{i+1}}{2} \right) (t_{i+1} - t_i)$$

where t is the time of each reading, y is the disease severity % on each reading date, $t_{i+1} - t_i$ is the number of days between two readings, and n is the number of readings.

Data on each phenotypic variable (disease incidence, gall number, gall weight, disease severity, and AUDPC) for pollinated and unpollinated ears were analyzed separately. Disease incidence is a binary variable because each plant is either diseased or not and is characterized by a binomial distribution based on the presence or absence of galls on the ear (Madden and Hughes 1995). Disease incidence data was fitted to a generalized linear model with a logit link, that is considered broader in scope than a standard analysis of variance (ANOVA) or linear probability regression model for a binary outcome (Madden et al. 2002). Disease incidence was modeled as a function of genotypes and treatments (pollinated and unpollinated).

Two-way ANOVA was used for gall number, gall weight, disease severity, and AUDPC to compare the mean effects of genotypes and treatments. The four outcome variables were modeled as a function of genotypes and treatment status. ANOVA determined the mean differences in gall number, gall weight, disease severity, and AUDPC between genotypes and treatment status. The Shapiro-Wilk test was used to test the normality of the data before proceeding with ANOVA. Means associated with each treatment and genotype, were separated using Tukey's HSD test when the ANOVA indicated significance at the 95% confidence level. R software version 4.0.3 was used to analyze the data (R Core Team 2021). In all experiments, the effect of each treatment and genotype was determined at a 5% level of significance, and all tests were two-tailed.

RESULTS

Effects of Pollination on Disease Incidence

The generalized linear model for the disease incidence in ear shoots demonstrated significant variation across the six genotypes and treatment status (**Table A.1**). The interaction terms between genotypes and treatment status were not significant ($P>0.05$). Therefore, interactions were not included in the model. The coefficients from the generalized linear model were in the logit scale and exponentiating the coefficients resulted in the Odds Ratio (OR) estimates of disease incidence with a given genotype and treatment.

When comparing the six genotypes, the odds of disease incidence were significantly lower in the two resistant maize-teosinte NILs (MT-NIL1 and MT-NIL2) at 5% ($P< 0.05$) level of significance for all four observation dates as compared to the B73 parent (reference group). The likelihood of disease incidence was not significantly different among the other four maize genotypes compared to B73 for all four observation dates. Additionally, the likelihood of disease incidence of ears was significantly lower ($P< 0.05$) for pollinated genotypes at 10, 14, and 21 DPI and significantly higher for unpollinated genotypes at the same time points. There was no significant difference in disease development between pollinated and unpollinated plants for MT-NIL1 at 7 DPI.

Effects of Pollination on Ear Gall Number and Gall Weight

Test of homogeneity of error variance demonstrated that the mean squares were not heterogenous for gall number and gall weight the summer of 2020 and 2021. Hence, separate data analysis was not performed. Instead, the data from the Summer of 2020 and 2021 were pooled together. A significant ($P< 0.001$) difference in gall number and gall weight was observed for genotypes and pollination (**Table A.2**). However, the genotypes × pollination interaction for disease severity did not show significant ($P=0.97$) variations. The effects of pollination on mean ear gall number at 21 DPI among maize genotypes and NILs are shown in **Figure A.2.** The mean ear gall number for pollinated MT-NIL1 and MT-NIL2 was significantly lower ($P<0.001$) than unpollinated NILs and three maize genotypes, when incoculated with *U. maydis*.

Conversely, the mean gall number was higher for unpollinated plants across all maize genotypes tested. Similar results were observed for ear gall weight (**Figure A.3**). The mean ear gall weight for both pollinated NILs was significantly lower ($P<0.001$) than unpollinated NILs, and maize genotypes. *U. maydis* ear incoluations resulted in less severe infection of the NIL pollinated ears, in comparison to the pollinated maize genotypes in terms of gall number and gall weight.

Effects of Pollination on the Area Under Disease Progress Curve and Disease Severity

Similar to the gall number and gall weight, ANOVA results were observed for AUDPC and disease severity for corn smut disease. Test of homogeneity of error variance demonstrated the mean squares were not heterogenous for AUDPC and disease severity. Hence, separate data analysis was not performed. Instead, the data were combined for summer 2020 and 2021. ANOVA revealed a significant ($P< 0.001$) difference in AUDPC and disease severity at 21 DPI

for both genotypes and pollination (**Table A.3**). However, the genotypes × pollination interaction did not show significant variations for AUDPC (P= 0.97) and disease severity (P= 0.99) at 5% level.

The pollinated and unpollinated ears exhibited a significant (P< 0.05) difference in disease severity at 21 DPI. The mean disease severity of pollinated MT-NIL1 (31.67 %) and MT-NIL2 (25.83 %) was significantly lower (P< 0.05) than unpollinated MT-NIL1 (43.33 %) and MT-NIL1 (40 %) (**Table A.4**). However, the mean disease severity for the ears of both pollinated NILs was not significantly different from each other. Further, the mean disease severity of both pollinated and unpollinated NILs was significantly lower than both pollinated and unpollinated susceptible maize genotypes. Conversely, the mean disease severity was significantly higher (P< 0.05) in unpollinated maize genotypes (1. B73-75.83 %; 2. H95- 73.75 %; 3. Golden Bantam- 70.83 %; 4. Mo17- 67.50 %). Overall, the mean disease severity of pollinated maize genotypes and NILs were lower than unpollinated maize genotypes and NILs. Disease severity was assessed at 21 DPI because this is the time period at which galls mature fully and form teliospores.

Similarly, the AUDPC values for ear galls of the four maize genotypes and two NILs are presented in **Table A.4**. When comparing all pollinated and unpollinated plants, significantly lower AUDPC values (P< 0.05) were observed for pollinated NIL1 (345.67%) and NIL2 (306.33). However, AUDPC values for both pollinated NILs were not significantly different from each other. The highest AUDPC values were observed for unpollinated B73 (778.33 %), followed by unpollinated H95 (740.33 %), Golden Bantam (714.67%), and Mo17 (688.33 %). Overall, AUDPC values forpollinated maize and NILs were lower than all unpollinated plants.

DISCUSSION

All pollinated genotypes inoculated with *U. maydis* demonstrated significantly less corn ear smut disease severity than the unpollinated genotypes. Therefore, pollination reduced susceptibility to *U. maydis* in both resistant and susceptible genotypes. For all phenotypic characteristics assessed, the pollinated NILs were more resistant to *U. maydis* than unpollinated NILs and other maize genotypes, including the B73 NIL parent. This is partially due to the genetic background of the six genotypes. The two NILs carry genes that contribute to the resistance phenotype (Chavan and Smith 2014), whereas the four maize genotypes do not carry any known genes for resistance to *U. maydis* and are sucsceptible. Therefore, variation in gall development observed for the resistant NILs and four susceptible maize genotypes was due to genotype (presence or absence of resistance genes) and pollination (pollinated: more resistant; unpollinated: less resistant). Moreover, the effects of pollination were more prominent in the resistant NILs, indicating pollination in the NILs improved resistance to *U. maydis*.

Why would the pollinated NILs be more resistant to *U. maydis* than the unpollinated NILs, when both pollinated and unpollinated NILs carry the same disease resistance genes. Bassetti and Westgate (1993 a, b) reported that tissues at the base of the ear silk began to collapse due to senescence at about one week after the silks emerged from maize ear shoots. The senescence of silks before pollination reduced the number of kernels per ear that hindered successful fertilization. This suggests pollination possibly blocks *U. maydis* from colonizing ovaries in a manner similar to the formation of the abscission layer. These studies suggest that pollination shortened the period that maize ears were susceptible to *U. maydis* infection and decreased the incidence of ears with galls more rapidly when silks were exposed to pollen than when silks were not exposed. The timing of silk emergence and pollen maturation is extremely

sensitive to environmental conditions in the field. This may account for the wide variation often observed when maize hybrids are tested for resistance to *U. maydis*.

The improved resistance of both resistant and susceptible maize lines to *U. maydis* after pollination holds some promise for the opportunity to better understand the effects of pollination on resistance and to enhanced resistance to *U. maydis* and other fungal pathogens. Consequently, more detailed investigations are needed to analyze variation of improved resistance after pollination, determine how pollination mediated improved resistance impacts resistance gene mediated resistance and to rule out any substantial trade-off effects that might be associated with these interactions.

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Table A.1. Estimates for corn smut disease incidence (DI) of ears from six maize genotypes inoculated with *Ustilago maydis*.

Variable	**DI 7 DPI**	**DI 10 DPI**	**DI 14 DPI**	**DI 21 DPI**
Genotypes (ref: B73)				
Golden Bantam	-0.14	-0.92	-0.92	-0.50
	(0.38)	(0.58)	(0.58)	(0.64)
H95	0.61	0.98	0.98	1.27
	(0.37)	(0.86)	(0.86)	(0.89)
Mo17	-0.21	-0.80	-0.68	-0.66
	(0.38)	(0.59)	(0.60)	(0.59)
MT-NIL1	-0.79	-1.23*	-1.24***	-0.81***
	(0.40)	(0.56)	(0.28)	(0.23)
MT-NIL2	-1.21**	-1.03***	-1.07***	-0.29***
	(0.44)	(0.27)	(0.31)	(0.07)
Treatments (ref: Control)				
Pollination	-0.16	-0.84**	-0.91**	-0.73***
	(0.23)	(0.32)	(0.32)	(0.17)
Intercept	-0.33	2.89***	2.94***	0.28
	(0.29)	(0.52)	(0.52)	(0.25)
N	360	360	360	720
AIC	452.68	294.98	290.63	681.02
BIC	479.88	322.18	317.83	722.23

DPI represents days post inoculation. *, ** and *** indicate not significantly different from 0 at 5%, 1% and less than 1% significance levels, respectively, based on t-tests of regression coefficients.

Table A.2. Analysis of variance by genotypes and pollination for gall number and gall weight of maize ears inoculated with *Ustilago maydis*.

Response variable	**Source of variation**	**Degree of freedom**	**F value**	**Pr(>F)**
Gall number	Genotypes	5	18.43	<0.001
	Pollination	1	34.47	<0.001
	Genotypes x Pollination	5	0.17	0.97
	Residuals	348	-	-
Gall weight	Genotypes	5	17.03	<0.001
	Pollination	1	29.11	<0.001
	Genotypes x Pollination	5	0.19	0.96
	Residuals	348	-	-

F value is a test statistics to determine if the variance between the means of the compared population is significantly different. Pr (>F) gives a P value where <0.001 indicates statistical significance at less than 1% level and otherwise insignificant.

Table A.3. Analysis of variance of the area under the disease progress curve and disease severity of maize ears 21 DPI with *Ustilago maydis*.

Response variable	Source of variation	Degree of freedom	F value	Pr(>F)
AUDPC	Genotypes	5	16.36	<0.001
	Pollination	1	21.42	<0.001
	Genotypes x Pollination	5	0.18	0.97
	Residuals	348	-	-
Disease severity at 21 DPI	Genotypes	5	19.02	<0.001
	Pollination	1	27.962	<0.001
	Genotypes x Pollination	5	0.13	0.99
	Residuals	348	-	-

AUDPC and DPI represent the area under disease progress curve and days post inoculation, respectively. F value is a test statistics to determine if the variance between the means of the compared population is significantly different. Pr (>F) gives a P value where <0.001 indicates statistical significance at less than 1% level and otherwise insignificant.

Table A.4. Effect of pollination on disease severity and area under the disease progress curve of maize ears of different genotypes.

Genotype	Treatment	AUDPC±SE	DS at 21 DPI±SE
B73	Control	778.33±49.61 [a]	75.83±4.40 [a]
	Pollination	631.67±49.04 [bcd]	58.75±5.43 [bc]
Golden	Control	714.67±59.84 [abc]	70.83±5.97 [ab]
	Pollination	559.33±63.71 [de]	55.42±6.47 [cd]
H95	Control	740.33±38.85 [ab]	73.75±4.34 [a]
	Pollination	652.00±35.93 [abcd]	55.42±3.15 [cd]
Mo17	Control	688.33±67.51 [abcd]	67.50±6.53 [abc]
	Pollination	585.33±44.35 [cde]	54.58±4.30 [cd]
MT-NIL1	Control	482.00±38.11 [ef]	43.33±4.05 [de]
	Pollination	345.67±55.04 [g]	31.67±5.11 [f]
MT-NIL2	Control	469.67±29.19 [ef]	40.00±3.19 [de]
	Pollination	306.33±46.85 [g]	25.83±4.24 [f]

Numbers indicate means ± standard errors. Within columns, least-squares means followed by the same letter are not significantly different based on Tukey's HSD test at the 95% confidence interval. AUDPC, DS, DPI and SE represent areas under disease progress curve, disease severity, days post inoculation and standard error, respectively.

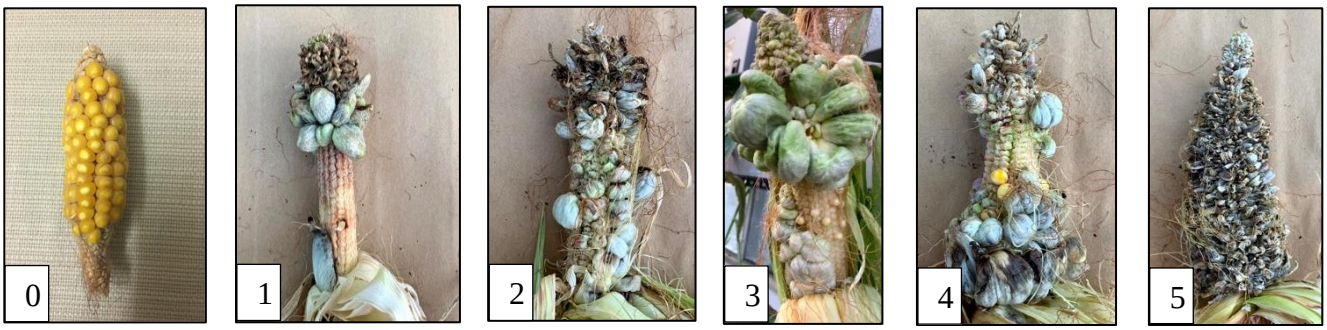

Figure A.1. Representation of ear gall disease severity rating scales.

The degree of disease severity was assigned as follows: 0 = no infection on ears, 1= 1-20% infected, 2 = 21-40% infected, 3 = 41-60% infected, 4 = 61-80% infected, and 5 = 81-100% infected.

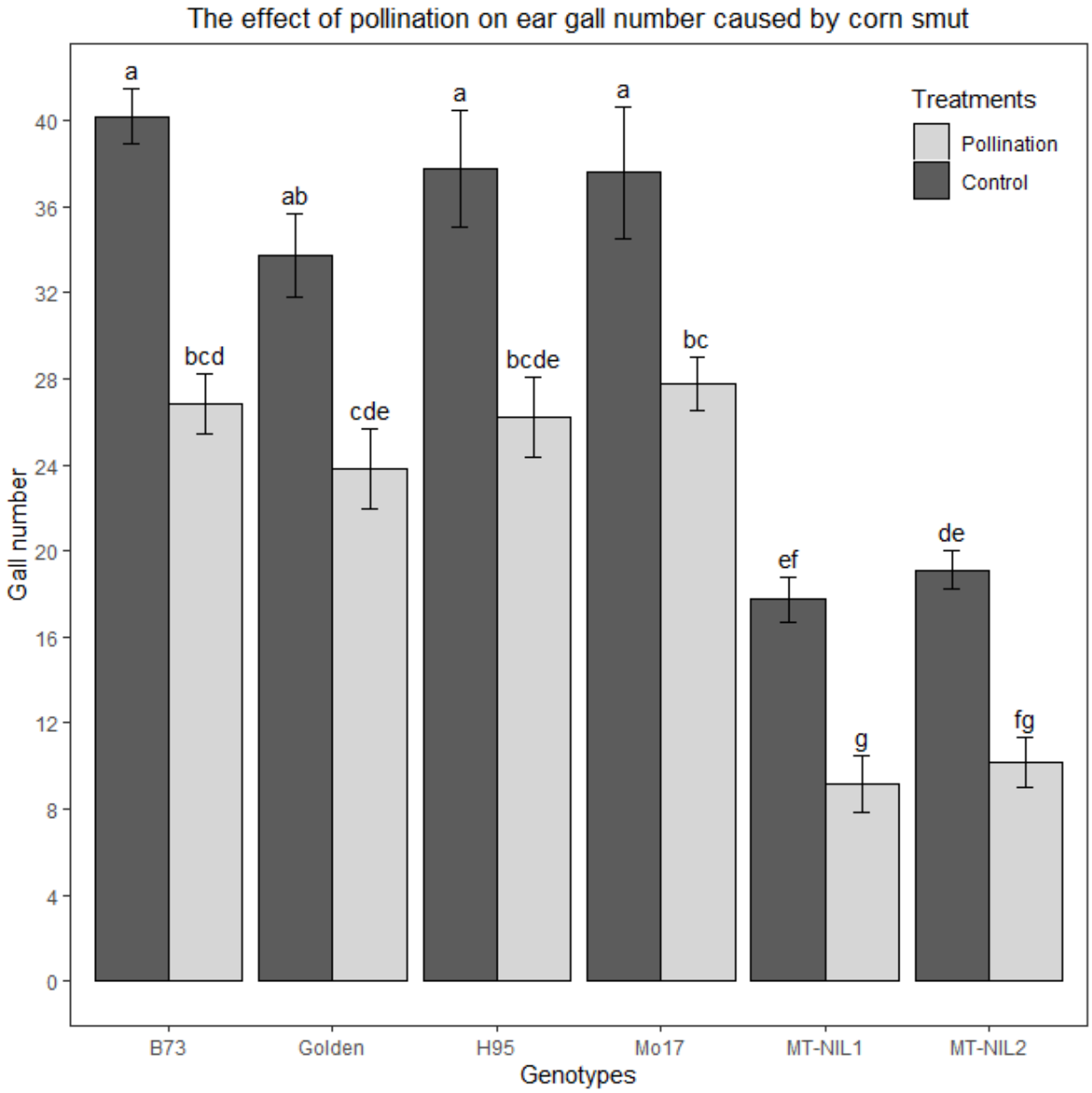

Figure A.2. Mean ear gall number for six maize genotypes with two treatments.

The x-axis represents four maize genotypes and two NILs. The y-axis represents the ear gall number. The figure legend corresponds to the bars in the figure and indicates the two treatments (pollinated and control). The same letter above the bars indicates no significant difference between ($P < 0.001$) between pollinated and control (unpollinated) experiments.

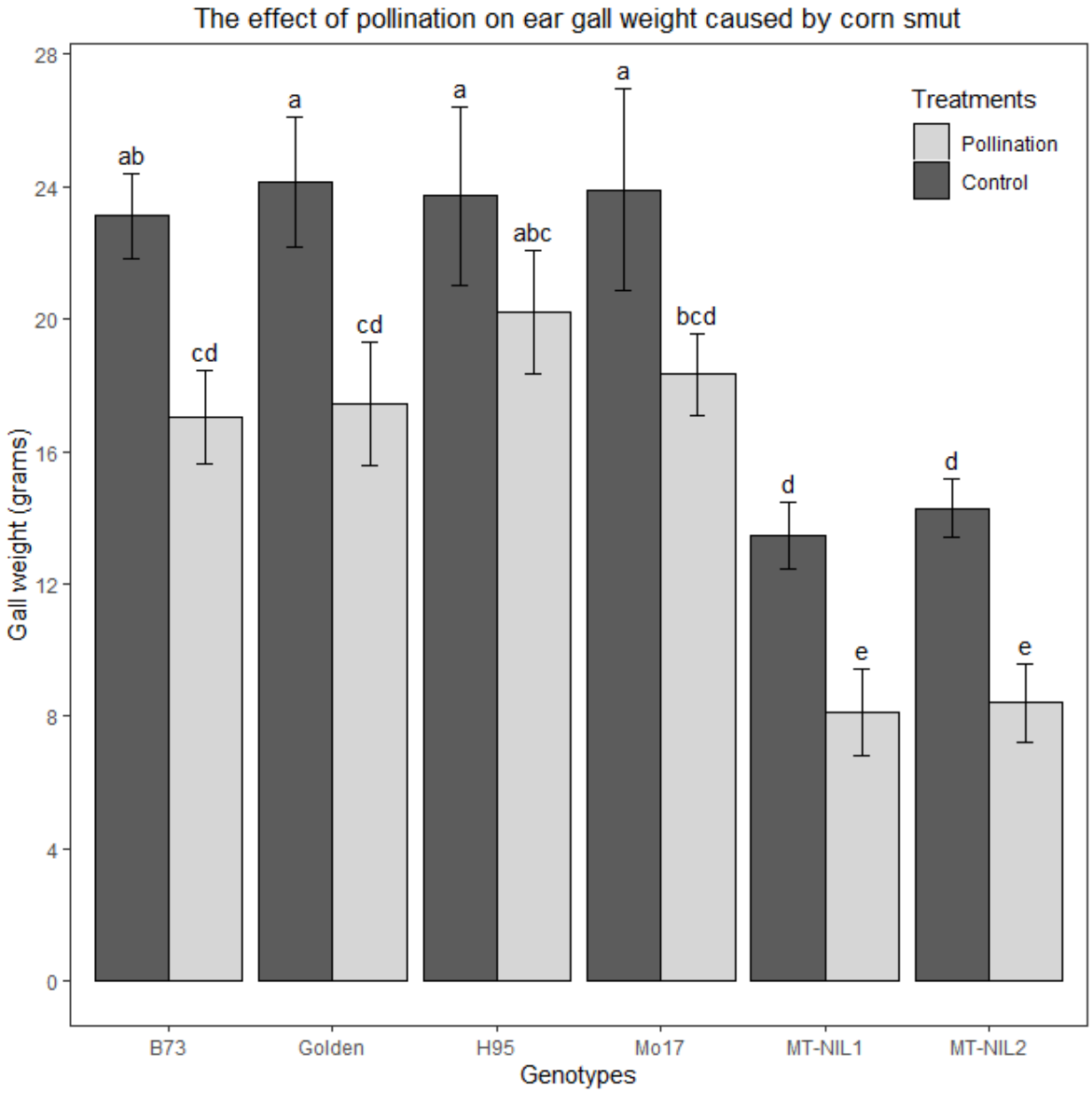

Figure A.3. Mean gall weight (grams) for six maize genotypes with two treatments.

The x-axis represents four maize genotypes and two NILs. The y-axis represents ear gall weight in grams. The figure legend corresponds to the bars in the figure and indicates the two treatments (pollinated and unpollinated/control). The presence of the same letter above the bars indicates no significant difference ($P < 0.001$) between pollinated and control (unpollinated) experiments.

APPENDIX B

PHENOTYPIC ANALYSIS OF MAIZE AND MAIZE-TEOSINTE RESISTANCE TO *USTILAGO MAYDIS*

ABSTRACT

The corn smut fungus *Ustilago maydis* is responsible for significant corn yield losses. It locally induces tumors and infects all aerial plant parts. The lack of resistant maize cultivars necessitates the identification of new sources of resistance. The near-isogenic lines (NILs) obtained from the cross between maize (B73) and teosinte (*Zea mays* ssp. *parviglumis*), have shown some degree of evidence of resistance to corn smut. Genetic analysis identified a 3.9 Mbp teosinte introgression on chromosome 9 in the resistant NILs. However, little is known about the differential response of maize genotypes and two NILs to different levels of *U. maydis* on the tassel and ear-gall development. Experiments in a controlled environment were carried out to investigate the resistance of four maize genotypes (B73, H95, Golden Bantam, and Mo17) and two maize-teosinte lines (NIL1 and NIL2) to treatment levels of *U. maydis* in ears and tassels. Four maize genotypes, two NILs, and three teosintes (*Zea mays* ssp. *parviglumis*, *Zea diploperennis*, and *Zea luxurians*) were also evaluated in the seedling stage. Plants were inoculated with 10^6 cells/ml of *U. maydis* at four treatment levels (1ml, 2ml, 3ml, and 5ml) in ears and tassels, while seedlings were inoculated with four treatment levels (100 µl, 200 µl, 300 µl, and 500 µl). Plants were phenotypically evaluated for: (1) disease incidence; (2) disease severity; (3) gall size; (4) gall number; and (5) gall weight to compare the resistance between maize genotypes and treatment levels. The two NILs (tassels and ears) were more resistant to *U. maydis* than the four maize inbred lines, including the B73 NIL parent. The phenotypic results supported the genetic data and indicated that the two maize-teosinte NILs carry the genes associated with resistance to *U. maydis* and were derived from the teosinte parent. We identified a correlation between the 3.9 Mbp teosinte introgression derived from the *Z. mays* ssp. *parviglumis* resistant donor parent and resistance to *U. maydis* in the maize-teosinte NILs.

Keywords: *Ustilago maydis*, *Zea mays*, Near-isogenic lines, Teosinte, Phenotypes, Corn smut, Disease severity, Disease incidence

INTRODUCTION

Maize (*Zea mays*) is one of the most significant staple crops worldwide. It is the most abundantly grown cereal with an annual production of 1,068.3 million metric tons from a total area of 185 million hectares (FAO 2021). Maize is used for food, animal feed, and biofuel purposes (Cassidy et al. 2013). The United States (US) is the world's largest maize producer, with 375.4 million metric tons produced annually accounting for 35% of the global maize production. The US plays a significant role in the world maize trade market, exporting around 19% of its annual production and accounting for $9.2 billion total export value in 2020 (USDA 2020). Maize production worldwide, including developing countries, is subjected to many abiotic and biotic stresses, seriously compromising yields. Among the menacing biotic stresses, corn smut caused by the fungus *Ustilago maydis* is a critical constraint, responsible for significant corn yield losses infecting all aerial parts and locally inducing tumors (Pataky and Snetselaar 2006). *U. maydis* has a limited host range infecting maize and a few teosinte genotypes (Pataky and Snetselaar 2006). *U. maydis* infects maize via several entry points such as silks, wounds caused by insect feeding, hail, wind, detasseling, and mechanical damage. The fungus colonizes maize plants locally by penetrating the cuticle and cell wall of the epidermal cells. The most prominent symptom is the formation of galls on the above-ground parts, mostly on seedlings, ears, tassels, stalks, nodal shoots, and midribs of leaves (Basse and Steinberg 2004; Kamper et al. 2006). The corn smut pathogen overwinters as teliospores in crop debris and soil, where it can remain viable for several years.

U. maydis has been reported to infect 1%-5% of plants within cultivated maize lines resulting in lower yield, and increased lodging, thereby favoring fungal growth (Immer and Christensen 1928; Christensen 1963; Pataky and Snetselaar 2006). Similarly, corn smut was reported to cause 0.3 million bushels of maize yield loss in 28 US corn-producing states and 1.8 million bushels of maize yield loss in Ontario, Canada in 2020 (Mueller et al. 2021). The majority of cultivated maize lines are susceptible to corn smut, while only a few intermediately resistant maize lines have been reported such as Zong 3 (Ding et al. 2008) and A188 (Baumgarten et al. 2007). No maize cultivars with hgh or complete resistance to *U. maydis* have been identified in large-scale management of corn smut (Smith 2011). Intermediately resistant maize cultivars can maintain yield losses limited to 2% (White 1999; Allen et al. 2011). However, due to the high value of maize grown in the US, a loss of 2% translates to nearly $1.0 billion losses (Allen et al. 2011).

Globalization of agriculture has resulted in crop plants with a narrow genetic base in areas far away from their centers of origin and distant from the pathogens that have co-evolved with them. These plants, therefore, 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). When new pathogen strains migrate to new locations where cultivated crops are grown, the plant genotypes are often susceptible, resulting in plant disease development dramatically reducing crop yield. Cultivated maize genotypes appear to have lost some of their direct defenses during selective breeding (Davila-Flores et al. 2013).

Although corn smut is one of the most important diseases affecting maize in developing countries, most studies overlook measuring yield loss quantitatively. Among all of the management practices such as sanitation, crop rotation, modification of fertilizers, seed

treatments, and fungicide application, the use of resistant varieties is the most practical and sustainable method to manage corn smut disease (Sherf and Macnab 1986; Pataky and Snetselaar 2006; Smith 2011). The significant economic loss incurred due to *U. maydis* and the lack of resistant maize cultivars indicated the need to identify new sources of resistance. Host resistance can be accomplished by exploiting the gene pool of wild maize progenitors and utilizing plant disease resistance genes in breeding programs (Smith 2011). In the early 1920s, wild progenitor species were introduced into several breeding programs to provide pest and/or disease resistance, abiotic stress tolerance, and improve the performance of the cultivated crops (Loskutov 1999). The use of wild species as a source of pest or disease resistance has been successful in crops such as sunflower, potato, tomato, banana, soybean, and wheat (Wilson et al. 1991; Wilson and Gates 1993; Rick and Chetelat 1995; Riggs et al. 1998; Hoisington et al. 1999; Escalante et al. 2002; Suszkiw 2005; Hajjar and Hodgkin 2007). As a result, wild progenitor species have provided a broad pool of potentially valuable genetic resources and have been used to improve modern agriculture.

Teosinte is a wild progenitor of maize and is the common name for the wild taxa of *Zea*. The genus *Zea* is further divided into two sections, *Luxuriantes* and *Zea Luxuriantes* comprises three species (*Zea diploperennis*, *Zea perennis*, and *Zea luxurians*) and four subspecies (*Zea mays* ssp. *huehuetenangensis*, *Zea mays* ssp. *parviglumis*, *Zea mays* ssp. *mexicana*, and *Zea mays* ssp. *mays*). Among these teosintes, *Zea mays* ssp. *parviglumis* is the immediate progenitor of maize and has been reported to cross readily with maize to produce fertile hybrids (Chavan and Smith 2014; Warburton et al. 2017). Crosses between teosinte and maize have transferred resistance to various crop diseases such as Striga, gray leaf spot, southern corn leaf blight, southern corn rust, and maize streak virus (Barry et al. 1992; Menkir et al. 2006; Wang et al.

2008; Yallou et al. 2009; Lennon et al. 2016). For example, *Zea mays* ssp. *parviglumis* exhibited resistance to *Colletotrichum graminicola*, the causal agent of anthracnose leaf blight (de Lange et al. 2014). *Zea diploperennis* introgressed into maize showed resistance to the chlorotic dwarf virus (Findley et al. 1982). Similarly, resistance from *Zea mays* ssp. *mexicana* has also been introgessed into maize for pathogens such as downy mildew and *Fusarium* (Maazou et al. 2017). Teosinte is a perennial grass that can grow in sub-tropical environments that lack winter freezes to keep the pathogen populations under control. It is capable of tolerating significant disease and insect exposure due to its evolutionary history span during which it has been exposed to numerous pathogens. Effective integration of resistance genes from teosinte into cultivated maize would decrease yield loss and reduce dependence on chemical inputs. Although comparative studies between maize and teosinte suggest teosinte species are shown to confer broad-spectrum resistance to several fungal pathogens, their potential to reveal resistance traits against the *U. maydis* pathogen in modern maize is poorly explored.

In this study we evaluated three teosinte wild progenitors (*Zea mays* ssp. *parviglumis*, *Zea diploperennis* and *Zea luxurians*), two resistant maize-teosinte NILs and four maize genotypes (B73, H95, Golden Bantam, and Mo17) for resistance to *U. maydis* at the seedling and adult stages. Plants were phenotypically evaluated for: 1) disease incidence, 2) disease severity, 3) gall number, and 4) gall weight to correlate the plant genotype, resistance phenotype and quantity of the relative *U. maydis* fungal biomass at different treatment levels. Only a few studies have established a relationship between corn genotype and response to *U. maydis* (Thakur et al. 1989). Screening maize and teosinte genotypes for resistance to *U. maydis* and statistical analysis of their heterogeneous responses will assist with identifying sources of resistance based on disease incidence and severity. We also screened a population of maize-teosinte introgression

NILs for resistance to *U. maydis*. The maize-teosinte introgression NILs were created by backcrossing ten different *Zea mays* ssp. *parviglumis* accessions into the maize B73 background, creating 900 teosinte introgression lines. The maize-teosinte introgression NILs are predominantly maize genetic backgrounds that produce normal ears and tassels and have the overall physiology of cultivated maize plants. Each line is near-isogenic (BC_4S_4) with an average of 4% *Zea mays* ssp. *parviglumis* from random parts of the genome. The 900 NILs have each been genotyped with ~768 SNPs. Therefore, the regions introgressed into each NIL are known. A "minimum tiling path" of 100 maize-teosinte NILs from one *Zea mays* ssp. *parviglumis* accession was created. The 100 maize-teosinte NILs were used for this work and contain almost the entire *Zea mays* ssp. *parviglumis* genome with each introgression line carrying a different single region from the *Zea mays* ssp. *parviglumis* genome. In light of identifying a practical and sustainable approach to managing *U. maydis,* the objective of this study was to identify new sources of resistance to *U. maydis*.

MATERIALS AND METHODS

Plant Genotypes, Sources and Maintenance

Large greenhouse and growth chamber studies were performed to evaluate resistance to *U. maydis*. Four maize genotypes (B73, H95, Mo17, and Golden Bantam), three teosinte genotypes (*Zea mays* ssp. *parviglumis*, *Zea luxurians*, and *Zea diploperennis),* and two maize-teosinte NILs derived from a maize-teosinte NIL population were used for the greenhouse study. Seed for the maize and teosinte genotypes were obtained from the USDA, US National Plant Germplasm System, USA. The four maize genotypes are economically significant inbreds and have not been evaluated for resistance to *U. maydis* at the seedling and adult stages. The three teosinte genotypes

were evaluated to identify other potential sources of resistance to *U. maydis* in a maize wild progenitor. The two maize-teosinte NILs were derived from a population created by crossing maize B73 to a *Zea mays* ssp. *parviglumis* genotype resulting in 900 maize-teosinte NILs (BC_4S_4). The maize-teosinte NIL population was selected for this study because the B73 recurrent parent is susceptible to *U. maydis,* while the *Zea mays* ssp. *parviglumis* donor parent served as a potential source of resistance to *U. maydis*.

Plants used for the adult plant resistance greenhouse assay were grown by planting six seeds in 27 pots for each of the ten genotypes. The six plants were thinned to three plants per pot after the fourth leaf stage to ensure the growth of at least three healthy plants. Plants used for the seedling resistance greenhouse assay were planted in flats. All plants were grown in SunGro Horticulture, Professional Growing Mix. A 20-20-20 liquid feed fertilizer was applied after seedling emergence,and watered as needed. The plants were grown in the greenhouse with day and night temperatures of 78°F and 72°F and day and night length of 16 hrs and 8 hrs, respectively. The greenhouse study was conducted at the South Milledge Greenhouse Facility, located at the University of Georgia (UGA) in Athens, Georgia, from March 2020 to July 2021.

One hundred NILs from the maize-teosinte NIL population were also screened for resistance to *U. maydis* in a growth chamber located at the UGA South Milledge Greenhouse Facility. Seeds were planted in flats, fertilized and watered as described for the greenhouse study. Plants were grown in a growth chamber with day and night environments of 28/20°C temperature and 12/12 hour 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 maintained in the same growth chamber, for a growth environment that was congruent across the study.

Ustilago maydis Inoculum Preparation

Wild type *U. maydis* strain 1/2 (mating type a1b1) and a near-isogenic strain 2/9 (mating type a2b2) were used for inoculations. Potato Dextrose Agar (PDA) plates were streaked separately with each *U. maydis* strain (a1b1 and a2b2) using a sterile loop. The *U. maydis* streaked PDA plates were placed in a 30°C incubator for two days to grow colonies. After two days at 30°C, single colonies were selected from the plates with a sterile toothpick. The toothpicks containing a single *U. maydis* colony for each strain were placed in a seperate test tube containing 3 ml of Potato Dextrose Broth (PDB) and placed in a 30°C shaker at 200 rpm for two days. Growth of the cultures was monitored for a cloudy appearance over the two day period. Cultures with a cloudy appearance were removed from the shaker after two days, and measured at OD_{600} to ensure the concentration of the cells were at an OD of 1.0 for optimal plant infection (Chavan and Smith 2014; Allen et al. 2011; Redkar and Doehlemann 2016). The *U. maydis* cell suspension cultures were brought to a final concentration of 1×10^6 cells/ml, with sterile water in a final 30 ml culture volume. Equal volumes of the *U. maydis* cell suspension from each strain were mixed prior to inoculation and used immediately.

Ustilago maydis Seedling Greenhouse Inoculation

Four maize genotypes (B73, H95, Mo17, and Golden Bantam), three teosinte genotypes (*Zea mays* ssp. *parviglumis*, *Zea luxurians*, and *Zea diploperennis)*, and two maize-teosinte introgressions NILs (ResNIL1 and ResNIL2) were used for this work. A preliminary greenhouse experiment was performed with 540 plants for each genotype to standardize the three *U. maydis* inoculation parameters: 1. inoculum concentration (cells/ml), 2. inoculation volume (µl for seedlings and ml for tassels and ears), and 3. inoculation method. The standardized *U. maydis* inoculation parameters were used for all greenhouse experiments.

Four maize genotypes (B73, H95, Golden Bantam, and Mo17), two NILs (NIL1 and NIL2), and three teosinte lines (*Zea mays* ssp. *parviglumis, Zea diploperennis*, and *Zea luxurians*) were examined to evaluate the phenotype of these cultivars at the seedling stage. Seeds were sown in 9 small flat containers. We used a two factor (9x4) completely randomized experimental design with nine cultivars and four treatment levels (100 µl, 200 µl, 300 µl, and 500 µl) of *U. maydis* cell suspension culture. Seedling infections were carried out at the three-leaf stage by injecting the *U. maydis* cell suspension culture into the stem of an experimental plant at a 90º angle. The culm of the seedlings was injected just above the soil line 10 days after planting. Each 10-day-old seedling was inoculated using a Covidien Monoject 3ml syringe (REF 8881513934) equipped with a 0.457 mm x 1.3 cm hypodermic sterile needle to the end of each 3 ml syringe. The same experiment was replicated thrice. The experimental unit was single seedlings with 10 seedlings in each replication for each cultivar-*U. maydis* treatment level. A total of 1,080 (9x4x10x3) seedlings were inoculated and evaluated for disease incidence and severity.

Ustilago maydis Tassel and Ear Greenhouse Inoculation

Tassel and ear experiments were also set up using two factors (6x4) experiments in a completely randomized design with four maize genotypes (B73, H95, Golden Bantam, and Mo17), plus two NILs (NIL1 and NIL2) and four treatment levels (1ml, 2ml, 3ml, and 5ml) of *U. maydis* cell suspension culture. We did not use teosinte for tassel and ear experiment because all three teosinte lines were completely resistant in the seedling stage and we assume that these teosinte lines would exhibit resistance in the inflorescence. The experimental unit was a plant planted in 10-inch diameter and 10-inch-tall containers. Plants with similar maturity were marked with tags in each replication. For the tassel inoculations, the leaf whorl was injected 3-10

days before the tassel emergence when the corn plants were 5-6 weeks old (Thakur et al. 1989). The primary ears were inoculated for the ear inoculation, and the corn ears were injected 4-6 days after silk emergence when silk had emerged 1 to 2 cm (Pataky and Chandler 2003). Each tassel and ear were inoculated using a Covidien Monoject 3ml syringe (REF 8881513934) equipped with a 18 G × 1 ½ inch hypodermic sterile needle to the end of each 3 ml syringe. The same experiment was replicated thrice. Each cultivar-treatment level of *U. maydis* consisted of 10 plants in each replication with a total of 720 (6x4x10x3) plants each for ears and tassels experiments separately.

Phenotypic Characterization and Disease Assessment Parameters

Disease incidence and disease severity were evaluated for seedlings, ears, and tassels. Disease assessment was carried out at 7, 10, 14, and 21 days post inoculation (DPI). Since the corn plant reached physiological maturity and began to senesce at 21 DPI, we used this reference day as a cut-off point for tassel and ear experiments. Previous studies have used several approaches to evaluate the disease incidence. We followed the binary disease assessment approach to assess whether or not a plant develops corn smut (Weiland et al. 2018). Disease incidence is evaluated on a presence (1) or absence (0) of galls in all three stages. Two phenotypic characteristics, disease incidence, and severity were assessed for the seedlings. Seedling disease severity was visually assessed using a 0-5 disease rating scale where 0 = uninfected, 1C = chlorosis, 1A = anthocyanin pigmentation, 2 = minor leaf galls, 3 = major leaf and stem galls, 4 = basal gall, and 5 = death of the plant (Gold et al. 1997; Smith and Chavan 2014; Allen et al. 2011).

Four phenotypic variables: (a) disease incidence, (b) disease severity, (c) gall number, and (d) gall weight were evaluated for ear and tassel. Disease incidence and severity of each

inoculated plant were measured at 7, 10, 14, and 21 DPI. Gall number and gall weight data were taken 21 DPI. The severity of tassel and ear disease was rated as the percentage of the tassel and ear covered with galls, respectively. Disease severity of tassel and ear galls was rated on a 0-5 disease rating scale where 0 = uninfected tassel and ears, 1 =1-20% infected, 2 = 21-40% infected, 3 = 41-60% infected, 4 = 61-80% infected, 5 = 81-100% representing death of the tassel/ears (modified from- Johnson and Christensen 1935; Pataky 1991; Pataky and Chandler 2003; Pataky and Snetselaar 2006; Schilling et al. 2014; Redkar and Doehlemann 2016). We took the midpoint of each rating interval as a measure of severity for the analysis.

Statistical Analysis of Disease Incidence and Severity

Data on phenotypic variables (disease incidence and severity from seedlings, ears and tassels and gall number and gall weight for ears and tassels) were analyzed separately. Disease incidence is a binary variable because each plant is either diseased or not and characterized by a binomial distribution (Madden and Hughes 1995). The data on the disease incidence was fit to a generalized linear model with a logit link, which is considered broader in scope than a standard analysis of variance (ANOVA) or linear probability regression model for a binary outcome (Madden et al. 2002). Disease incidence was modeled as a function of cultivars and four concentrations of *U. maydis* cell suspension inoculation treatments.

We used a two-way ANOVA for gall number, gall weight, and severity to compare the mean effects of cultivars and treatments. These outcome variables were modeled as a function of cultivars and treatment doses of *U. maydis*. ANOVA determines the mean differences in gall number, gall weight, and severity between cultivars and treatment levels. The Shapiro-Wilk test was used to test the normality of the data before proceeding with ANOVA. Means associated with each treatment and cultivars were separated using Tukey's HSD test when the ANOVA

indicated significance at 5% levels. We used R software version 4.0.3 to analyze the data (R Core Team 2021). In all experiments, the effect of each treatment and cultivar was determined at a 5% level of significance, and all tests were 2-tailed.

RESULTS

Chavan and Smith (2014) inoculated and phenotyped one-hundred maize-teosinte NILs lines to identify new sources of resistance against *U. maydis*. These lines were created by backcrossing 10 different teosinte (*Zea mays* 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 contains almost the entire teosinte genome with each introgression line carrying a single different region from the teosinte genome.

Out of the 100 NILs, only two NILs were reported to be resistant to the corn smut disease at the seedling stage, and the remaining 98 NILs were found to be susceptible (Chavan and Smith 2014). Based on the genotypic evaluation, these two resistant NILs were found to have a 3.9 Mbp teosinte introgressed region in the short arm of chromosome 9, potentially responsible for the resistant phenotype. Thus, we hypothesize that teosinte can likely be used as a source for improving resistance in modern, annual temperate maize against *U. maydis*.

Disease Assessment in Seedlings

The results from the generalized linear model for the disease incidence in seedlings showed significant variation across cultivars, treatment levels, and exposure time to *U. maydis*

(**Table B.1)**. The interaction terms between cultivars and treatment levels were not significant (P>0.05); therefore, interactions were not included in the model. The coefficients from the generalized linear model are in the logit scale, and exponentiating the coefficients results in the Odds Ratio (OR) estimates of disease incidence with a given cultivar and treatment levels. Among the genotypes, the odds of disease incidence was found to be significantly lower in all three teosinte lines (*Zea mays* ssp. *parviglumis*, *Zea luxurians*, and *Zea diploperennis*) and two NILs (NIL1and NIL2) at a 5% (P< 0.05) level of significance for all four observation dates as compared to the parent B73. For instance, the ORs of disease incidence at 7, 10, 14, and 21 DPI associated with the NIL2 were 0.73, 0.69, 0.69, 0.69, respectively, indicating that NIL1 was less likely to develop smut gall after disease inoculation compared to its parent B73. The likelihood of disease incidence was not significantly different among other maize genotypes compared to B73 for all four observation dates. Additionally, among the four treatment levels, the likelihood of disease incidence in seedlings was significantly higher for 200 µl at 10, 14, and 21 DPI, and significantly higher for 300 µl and 500 µl relative to 100 µl for all observation dates (7, 10, 14 and 21 DPI).

Figure B.1 presents the mean disease severity in cultivars along with different observation dates. The severity of corn smut varied with cultivars and DPI. The severity at 7 DPI was largely similar and below 10% among all cultivars. As DPI increased, higher disease severity was observed for all maize genotypes and NILs. The disease severity with the most prominent increment being observed in susceptible B73 and H95. High disease severity suggests that B73 and H95 seedlings are highly susceptible to corn smut. The disease severity in teosintes was largely constant. The disease progress curve for maize genotypes, NILs, and teosinte showed similar patterns among themselves.

Further, the mean disease severity in seedlings of both the NILs was significantly lower (around 20%) compared to the parent B73 and other genotypes. However, these NILs themselves were not significantly different from each other. The mean disease severity in three teosinte lines, including the other NIL parent, *Zea mays* ssp. *parviglumis*, was significantly lower (around 10%), indicating the wild teosinte are resistant to the pathogen *U. maydis* in the seedling stage. Results suggest that teosinte seedlings are the most resistant to *U. maydis,* followed by the two NILs.

Disease Assessment in Ears

Similar to disease incidence in seedlings, odds of disease incidence in corn ear were significantly lower in both NILs relative to B73 for all observation dates at a 5% level of significance (**Table B.2**). The lower odds of gall development after *U. maydis* inoculation indicate that the NILs ears were more resistant to fungal infection. Among the treatment levels, the likelihood of disease incidence in the ear was significantly higher for 2 ml at 7 DPI and 3 and 5ml for all other observation dates relative to 1ml. Higher disease incidence indicates more new cases of ear galls development as the treatment level of *U. maydis* increases. NIL1 showed more resistance to disease incidence than NIL2, 0.29 vs. 0.34 times the odds of disease development in parent B73.

The effects of treatment levels and maize genotypes/ NILs on mean ear gall number at 21 days after *U. maydis* injection are shown in **Figure B.2.** The mean gall number was higher when cultivars were inoculated with 5ml *U. maydis* across all maize genotypes/NILs. Among the cultivars, the mean ear gall number in NILs was significantly lower ($P<0.001$) (around 17 counts) at 5ml treatment than B73. Results indicate that *U. maydis* causes less severe infections in ear of NILs than other maize genotypes in terms of gall number. Similar results were observed

in ear gall weight (**Figure B.3**). The mean ear gall weight in both the NILs was significantly lower ($P<0.001$) (around 10 grams) at 5ml treatment than the parent B73 and other genotypes, indicating *U. maydis* causes less severe infections in the ear of NILs.

Regardless of treatment levels, the ear disease severity increased rapidly over DPI for all maize genotypes/NILs, with the most prominent increment being observed among maize genotypes (**Figure B.4**). The H95, B73, and Mo17 had higher disease severity, on average, more than 40% severity was reported at 21 DAI. The mean disease severity in ears of both the NILs was significantly lower ($P<0.001$) (around 35%) as compared to mean disease severity in ears of B73 and other genotypes. However, these NILs themselves were not significantly different from each other. Since resistance to *U. maydis* is a quantitative trait, this type of resistance doesn't block/eliminate the infection in NILs. Still, it reduces the disease due to reduced pathogen fitness such as colonization, reproduction, and transmission. The lowest mean ear disease severity was observed for both NILs compared to other genotypes at all observation dates.

Disease Assessment in Tassel

The effects of genotypes and *U. maydis* treatment levels on tassel disease incidence are presented in Table 3. Estimates for disease incidence in the tassel for 7, 10, 14, and 21 DPI are given in columns 2-5, respectively. Just like seedling and ear, the odds of tassel disease incidence were significantly ($P<0.001$) lower in both NILs, while that in Golden Bantam was significantly higher ($P<0.01$) compared to B73 for all observation dates. The lower odds of disease development imply that the NILs' tassels are resistant to fungal infection, suggesting the occurrence of new cases of tassel galls was significantly less in NILs than other genotypes. However, among the three treatments, the likelihood of disease incidence in tassel was significantly higher for 5ml relative to 1ml for 10 DPI, 14 DPI, and 21 DPI. Results indicate that

the occurrence of new cases of tassel galls increases as the treatment level increases. These findings are consistent with the results from ear inoculation.

The mean gall weight per tassel increases with the augmented treatment levels (**Figure B.5**). The mean tassel gall weight in two NILs was significantly lower (almost zero gram) than it was in parent B73 and other genotypes. In addition, no significant differences in mean tassel gall weight were observed across four treatment levels in both NILs. This result indicates that the tassels in NILs were resistant to *U. maydis* compared to other genotypes. The observed mean difference in tassel gall weight was not different from zero among 1, 2, and 3 ml *U. maydis* inoculation for all maize genotypes. However, gall weight increased significantly with a 5 ml treatment level for all genotypes. The highest tassel gall weight was observed in Golden Bantam, Mo17 and H95 at 5 ml *U. maydis* treatment level. Also, we found that only a higher amount of inoculum affects seed development in ear (5ml in this case), whereas the lower treatment levels provided a good seed set in NILs compared to other genotypes. The tassel gall number also varied significantly among the maize genotypes/NILs and treatment levels, except for the treatment levels within the NILs (**Figure B.6**). The maize genotype, H95, had produced a larger number of tassel galls at the 5 ml treatment level, followed by Golden Bantam and Mo17. **Figure B.7** shows the tassel disease severity from the tassel inoculation. The tassel disease severity graph was similar to that of ear disease severity. As in the ear, the disease severity in both NILs was significantly lower (almost 0%) than those in B73 and other genotypes. These two NILs were not significantly different from each other. The lower disease severity further provides evidence that two NILs were more resistant to the fungal pathogen *U. maydis* in the tassel than other genotypes. More resistance means NILs can form pollen and fertilization can take place.

DISCUSSION

Since host resistance is the only practical and sustainable method for managing corn smut disease, the lack of resistant maize cultivars necessitates the study on identifying new sources of resistance to *U. maydis*. To this end, only limited information is available on the evaluation of corn genotypes for resistance to *U. maydis* (Thakur et al. 1989; Chavan and Smith 2014). This study provides the first quantitative report on the phenotypic analysis of resistance to *U. maydis* in the tassels and ears of the two NILs and four maize genotypes and in addition seedlings of three teosinte lines. The seedling, ear, and tassel inoculation methods effectively produced a visible smut disease incidence and severity in genotypes that allow us to examine the differentiated response of *U. maydis* across these genotypes and treatment levels. In seedling experiments, three teosinte lines; *Zea mays* spp. *parviglumis*, *Zea luxurians*, and *Zea diploperennis* were found to have the lowest disease incidence and severity value, followed by the two NILs. These results show teosinte demonstrated complete resistance in the seedling stage, followed by NILs. In the ears and tassels stages, both the NILs were resistant compared to the four maize genotypes based on the disease incidence, gall weight, gall number, and disease severity.

Previous studies have shown that identifying crops' resistance to various biotrophic pathogens helps minimize yield losses (Mangelsdorf and Reeves 1957; Crepet and Feldman 1991; Iltis 1997). The phenotypic evaluation is generally considered the first step in identifying crop resistance. Screening plant phenotypic characteristics, whether it is resistant or susceptible, is primarily determined by the ability of the pathogen to gain access to the plant tissue. One of the important challenges to studying the genetic nature and evaluation of resistance to *U. maydis* is hindered by the lack of precise, reliable, and repeatable inoculation methods (Pope and McCarter

1992; Kakueinezhad et al. 2017). We conducted preliminary experiments to select an appropriate pathogen inoculation method, concentration, and amount of the inoculum. This approach efficiently delivers the pathogen in the plant seedlings and meristematic tissue that would consistently induce the formation of galls and enable the rapid identification of the resistant genotypes (Thakur et al. 1989; Chavan and Smith 2014).

Recognizing resistance against pathogens is best accomplished under field conditions. However, multiple pathogens often infect maize, making it very difficult to screen or work against a single pathogen under field conditions. Owing to non-uniform infections and the impossibility of controlling environmental conditions in the field, one of the approaches was to conduct experiments in greenhouse conditions, which made it possible to reduce the confounding factors and differentiate plants for resistance to *U. maydis* (Smith and White 1988; Tymon et al. 2016; Kakueinezhad et al. 2017). To achieve the same resistant phenotype among plants from the same line, we replicate the same experiment thrice on seedlings, tassels, and ears (Brefort et al. 2005; Doehlemann et al. 2008).

Several methods have been developed for seedling inoculation, such as dip inoculation, pipetting the pathogen cell suspension culture into the whirl, and needle injection (Gottwald and Graham 1992; Freeman and Rodriguez 1993; Posada et al. 2007; Estrada et al. 2012). Among them, the needle injection of *U. maydis* cell suspension culture, which was adopted in this study, has been described as a consistent and reliable method to facilitate pathogen penetration and appressoria formation (Chavan and Smith 2014). Further, Gold et al. (1997) and Allen et al. (2011) have reported needle inoculation protocol to demonstrate a range of phenotypes for maize and teosinte seedlings that can be used to determine the best concentration for the pathogen cell suspension culture resulting in consistent plant phenotypes. We used a 10^6 cells/ml

concentration, which resulted in a consistently good infection in the seedling stage (Chavan and Smith 2014).

According to du Toit and Pataky (1999), disease incidence in maize ears inoculated via silk channel has been shown to increase rapidly between 10^3 and 10^5 sporidia/ml. However, escapes ranged from 15 to 80% at 10^3 to 10^4 sporidia/ml and from 5 to 40% at 10^5 to 10^6 sporidia/ml. Thus, concentrations between 10^5 and 10^6 sporidia/ml have been shown to minimize variation; therefore, we selected a concentration of 10^6 sporidia/ml for inoculating maize ears. However, inoculum concentrations $>10^6$ sporidia/ml have been reported to cause additional ear gall development (du Toit and Pataky 1999). The inoculum concentration must be controlled when screening for resistance to common smut with the silk channel method. Another study by du Toit and Pataky (1999) has also shown the variation among people inoculating has led to the inappropriate measurement of the corn smut incidence and severity. For example, the incidence of ears with galls differed among people inoculating by as much as 30%. The study also noted that the incidence and severity ratings were lower for people inexperienced at inoculating with *U. maydis* than experienced people. In this study, to avoid confounding variations, a single person having a good experience performed all the greenhouse inoculation experiments. In addition, a revised disease resistance rating scale was used to screen these genotypes and detect corn smut development.

For tassel inoculation, concentrations of 10^6 cells/ml (Thakur et al. 1989) have been shown to provide good infection; therefore, we selected a concentration of 10^6 cells/ml for inoculating maize tassels. Thakur et al. (1989) observed that the tassel florets were not infected after emergence from the leaf whirl. These results were consistent with the results of our preliminary experiment. Therefore, we inoculated tassels 3-10 days before tassel emergence. The

exact time and point of inoculating tassel inflorescence were identified when leaf sheath near the terminating shoot overlaps (Walbot and Skibbe 2010). The whirl of the immature leaf bases above the shoot apical region is more readily compressed and does not have a solid center. This region is considered the best site for inoculation as it contains the vegetative shoot apical meristem, which later develops into the tassel inflorescence (Danilevskaya et al. 2008; Redkar and Doehlemann 2016). The tassel inoculation protocol involved injecting different treatment doses in three different positions along with the immature tassel. For example, for 3ml *U. maydis* cell suspension culture, each position would get 1ml of the culture. Almost all visible florets on tassels of B73, H95, Golden Bantam, and Mo17 were converted to tumorous growth. In line with the previous study, we observed that the palea, lemma, anthers, and stamens in the tassels were converted to tumors (Walbot and Skibbe 2010). In a few NILs, only a portion of the tassel with 3ml and 5ml *U. maydis* treatment levels were found to have organs converted to tumors. However, in both the NILs, most of the tassel florets did not have galls and the tassels continued normal development and shed fertile pollen. Later, 21 days post-inoculation, we counted each glume with tumors as a single gall to determine the mean gall number.

For the ear inoculation, Pataky and Chandler (2003) and Pataky et al. (1995) identified that a silk-channel inoculation procedure resulted in a significantly higher incidence of ear galls than natural infection. Similarly, Pope and McCarter (1991) reported sporidial injection into cob tissue at the mid silk stage to be successful. Regarding the severity of ear galls, ears inoculated 4-6 days after the mid-silk growth stage have been found to be the highest (Pataky and Chandler 2003). For example, du Toit and Pataky (1999a) observed that the incidence of ears with galls differed by as much as 20% when plants were inoculated 3 days apart and by as much as 70% when inoculation was delayed by a week. It is because a large percentage of ears escaped

infection when plants were infected 2 days after mid silk, probably because silks have not emerged from some plants. In addition, when plants were inoculated later than 7-8 DPI, severity was found to be low, apparently because silks began to senesce (Bassettii and Westgate 1993a, b). These results were consistent with the results of our preliminary experiment. Therefore, we inoculated the ears around 4-6 DPI. Kernel infection of corn by *U. maydis* supposedly occurs due to the growth of dikaryotic mycelium through the silks (Shurtleff 1980).

We found a good infection in all the ears of four maize genotypes, where each kernel was converted to tumors for all treatment levels of *U. maydis*. However, the kernels of both the NILs did not completely convert to tumors at lower treatment levels such as 1ml and 2ml. Ear galls were relatively small and spongy and were protected by husk leaves until 8 to 12 days after the mid silk stage. Smut galls usually erupt through husk leaves 12 to 18 days after mid silk. Galls continued to enlarge until 19 to 21 days after mid silk (Pataky 1991). Sporulation of *U. maydis* also increased during this time, and most ear galls were too mature and of bad quality after 21 DPI. The weight of both the tassel and ear galls was taken 21 DPI via CGOLDENWALL High Precision Scale 10 kg 0.1g Digital Accurate Electronic Balance Lab Scale.

Although the pathogenesis of *U. maydis* on corn is well studied (Christensen 1963), the epidemiology of corn smut is not understood completely. Walbot and Skibbe (2010) suggests that fungal factors and host responses to the pathogen can impact ears and tassels. The other possible reason would be the physiological and morphological structure of the host that can have an impact on colonization of *U. maydis*. Additionally, *U. maydis* interacts differently with vegetative and reproductive organs, possibly by expressing different genes in specific plant parts. Therefore, what types of cells can be colonized by *U. maydis* and in what specific ways host status influences the pathogen's success is still unknown. Several investigators have observed

that for some corn genotypes, gall formation is specific for certain tissues, such as ears, tassels, or nodal shoots (Christensen 1963). We agree with this study because most of the gall formation in NILs was in ears compared to seedlings and the tassel stage. However, the mechanism behind it is not well understood. Since B73 is susceptible to *U. maydis*, the resistance NILs might have major resistance genes (R-genes) or pathogenesis-related (PR) proteins derived from the teosinte parent that contribute to resistance against corn smut disease.

In conclusion, the findings from this study demonstrated the presence of strong *U. maydis* resistance in both the NILs, which further verifies that wild parent *Zea mays* ssp. *parviglumis* is a very good source of *U. maydis* resistance that should be exploited in modern maize breeding programs. Genotypic analysis of both the NILs will be fruitful in identifying the mechanism behind resistance and provide the foundation for future tests of the hypothesis that the specific R-genes and PR proteins derived from the resistant parent *Zea mays* ssp. *parviglumis* are contributing to the resistance in both the NILs. Currently, comparative genome analysis of *Zea mays* ssp. *parviglumis*, B73, and a nested association mapping population have identified genes present only in the *Zea mays* ssp. *parviglumis* parent, suggesting these genes may contribute to the resistant phenotype in both NILs. To make gene predictions and assess the expression of candidate genes, RNA-sequencing is ongoing for the two NILs, B73 and *Zea mays* ssp. *parviglumis* inoculated with *U. maydis*. These data will identify candidate genes expressed in two NILs in the 3.9 Mbp region of chromosome 9 derived from the teosinte parent and potentially confer resistance to *U. maydis*. This research is crucial in managing *U. maydis* in cultivated maize lines. Overall, this work will have significant implications in characterizing durable resistance mechanisms and developing novel strategies to manage *U. maydis*, more specifically, in developing countries. R-genes and PR proteins identified and characterized from

the resistant NILs can help improve resistance in modern, annual temperate maize against *U. maydis*.

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Table B.1. Estimates of *Ustilago maydis* incidence for seedlings from four maize genotypes, two NILs, and three teosinte lines

	Incidence 7 DPI	Incidence 10 DPI	Incidence 14 DPI	Incidence 21 DPI
Genotypes (ref: B73)				
Golden	-0.43	-0.45	-0.41	-0.41
	(0.27)	(0.29)	(0.29)	(0.29)
H95	0.38	-0.09	-0.09	-0.09
	(0.26)	(0.29)	(0.29)	(0.29)
Mo17	-0.21	-0.37	-0.37	-0.29
	(0.27)	(0.29)	(0.29)	(0.29)
NIL1	-0.76 **	-0.48***	-0.48***	-0.48 ***
	(0.28)	(0.8)	(0.8)	(0.9)
NIL2	-0.32***	-0.37 ***	-0.37***	-0.37***
	(0.7)	(0.9)	(0.9)	(0.9)
Zea diploperennis	-1.19 ***	-0.99 ***	-0.88 **	-0.88 **
	(0.30)	(0.28)	(0.28)	(0.28)
Zea luxurians	-0.76 **	-0.92 **	-0.88 **	-0.92 **
	(0.28)	(0.28)	(0.28)	(0.28)
Zea mays ssp.	-0.80 **	-1.16 ***	-1.16 ***	-1.16 ***
parviglumis	(0.28)	(0.28)	(0.28)	(0.28)
Treatments (ref: 100 µl)				
200 µl	0.33	0.43 *	0.40 *	0.45 *
	(0.20)	(0.18)	(0.18)	(0.18)
300 µl	0.61 **	0.66 ***	0.66 ***	0.68 ***
	(0.19)	(0.18)	(0.18)	(0.18)
500 µl	0.85 ***	1.25 ***	1.24 ***	1.25 ***
	(0.19)	(0.19)	(0.19)	(0.19)
Intercept	-0.72 **	0.47 *	0.48 *	0.46 *
	(0.22)	(0.23)	(0.23)	(0.23)
N	1080	1080	1080	1080
AIC	1333.66	1379.90	1378.25	1375.12
BIC	1393.47	1439.72	1438.06	1434.94

Table B.2. Estimates of *Ustilago maydis* incidence for ears from six genotypes and four treatment levels.

	Incidence 7 DPI	Incidence 10 DPI	Incidence 14 DPI	Incidence 21 DPI
Genotypes (ref: B73)				
Golden	-0.08	-0.61	-0.56	-0.56
	(0.28)	(0.32)	(0.32)	(0.32)
H95	0.30	-0.06	-0.06	-0.06
	(0.28)	(0.34)	(0.34)	(0.34)
Mo17	-0.04	-0.06	0.06	0.12
	(0.28)	(0.34)	(0.34)	(0.35)
NIL1	-0.98 **	-1.25 ***	-1.25 ***	-1.25 ***
	(0.31)	(0.32)	(0.32)	(0.32)
NIL2	-1.72 ***	-1.08 ***	-1.09 ***	-1.08 ***
	(0.37)	(0.32)	(0.32)	(0.32)
Treatments (ref: 1 ml)				
2 ml	0.69 **	0.20	0.15	0.20
	(0.26)	(0.22)	(0.22)	(0.22)
3 ml	1.52 ***	0.94 ***	0.90 ***	0.87 ***
	(0.25)	(0.24)	(0.24)	(0.24)
5 ml	0.18	2.57 ***	2.63 ***	2.63 ***
	(0.27)	(0.35)	(0.37)	(0.37)
Intercept	-1.22 ***	0.79 **	0.82 **	0.80 **
	(0.26)	(0.27)	(0.27)	(0.27)
N	720	720	720	720
AIC	791.92	752.42	743.58	742.85
BIC	833.13	793.63	784.79	784.07
Pseudo R2	0.18	0.22	0.23	0.23

Table B.3. Estimates of *Ustilago maydis* incidence for tassels from six genotypes and four treatment levels.

	Incidence 7 DPI	Incidence 10 DPI	Incidence 14 DPI	Incidence 21 DPI
Genotypes (ref: B73)				
Golden	0.83 **	1.45 ***	1.86 ***	1.86 ***
	(0.32)	(0.33)	(0.36)	(0.36)
H95	0.79 *	0.11	0.97 **	0.97 **
	(0.32)	(0.27)	(0.30)	(0.30)
Mo17	0.58	0.59 *	0.73 *	0.73 *
	(0.33)	(0.28)	(0.29)	(0.29)
NIL1	-1.71 **	-2.60 ***	-2.59 ***	-2.59 ***
	(0.57)	(0.35)	(0.35)	(0.35)
NIL2	-1.28 **	-2.17 ***	-2.16 ***	-2.16 ***
Treatments (ref: 1 ml)				
	(0.49)	(0.32)	(0.32)	(0.32)
2 ml	-0.09	-0.06	-0.32	-0.32
	(0.30)	(0.25)	(0.27)	(0.27)
3 ml	0.09	0.23	-0.11	-0.11
	(0.29)	(0.26)	(0.27)	(0.27)
5 ml	0.54	1.33 ***	1.08 ***	1.08 ***
	(0.28)	(0.28)	(0.29)	(0.29)
Intercept	-1.82 ***	0.08	0.28	0.28
	(0.31)	(0.25)	(0.25)	(0.25)
N	720	720	720	720
AIC	625.91	737.14	681.02	681.02
BIC	667.12	778.36	722.23	722.23

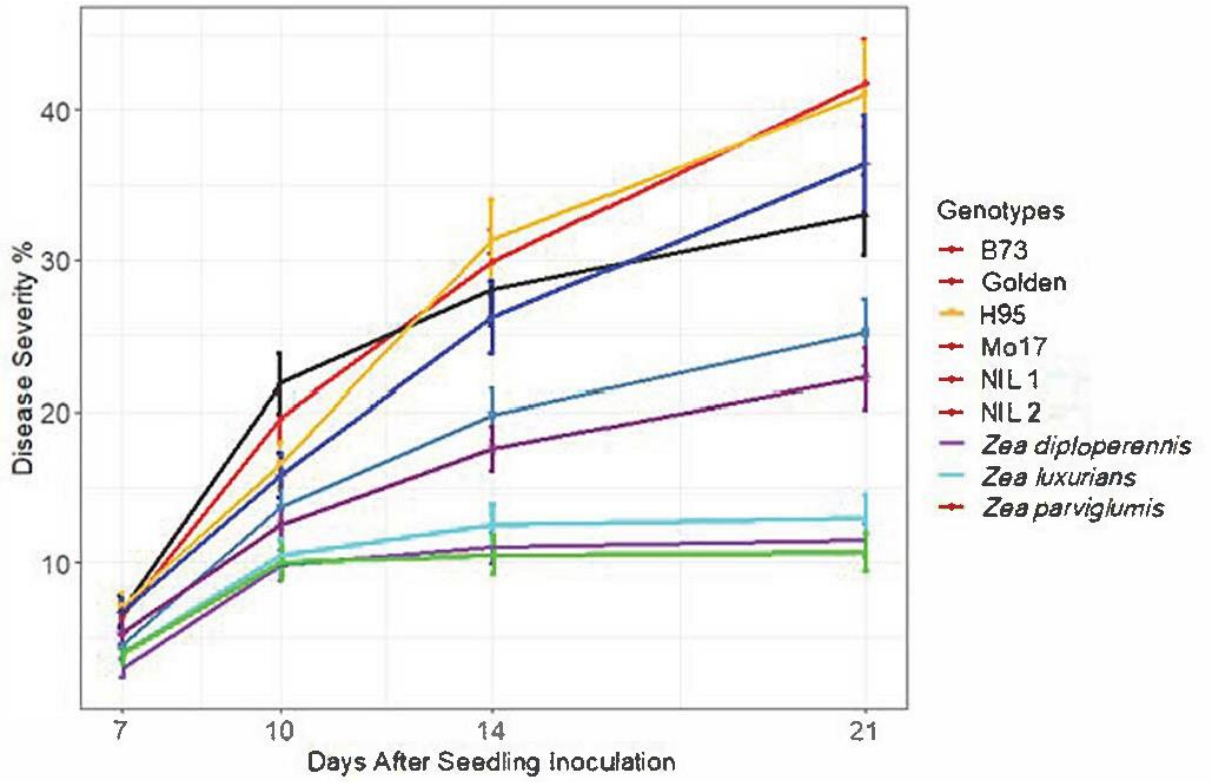

Figure B.1. Seedling disease severity for six genotypes at 7, 10, 14, and 21 days post-inoculation. The disease severity was estimated based on visual assessment on a disease rating scale of 0-5. The x-axis represents days post inoculation, and the y-axis represents the percentage of disease severity in seedlings. Different colored lines represent four maize genotypes, two NILs, and three teosintes. Means of disease severity in both NILs and teosinte lines were significantly lower than maize genotypes.

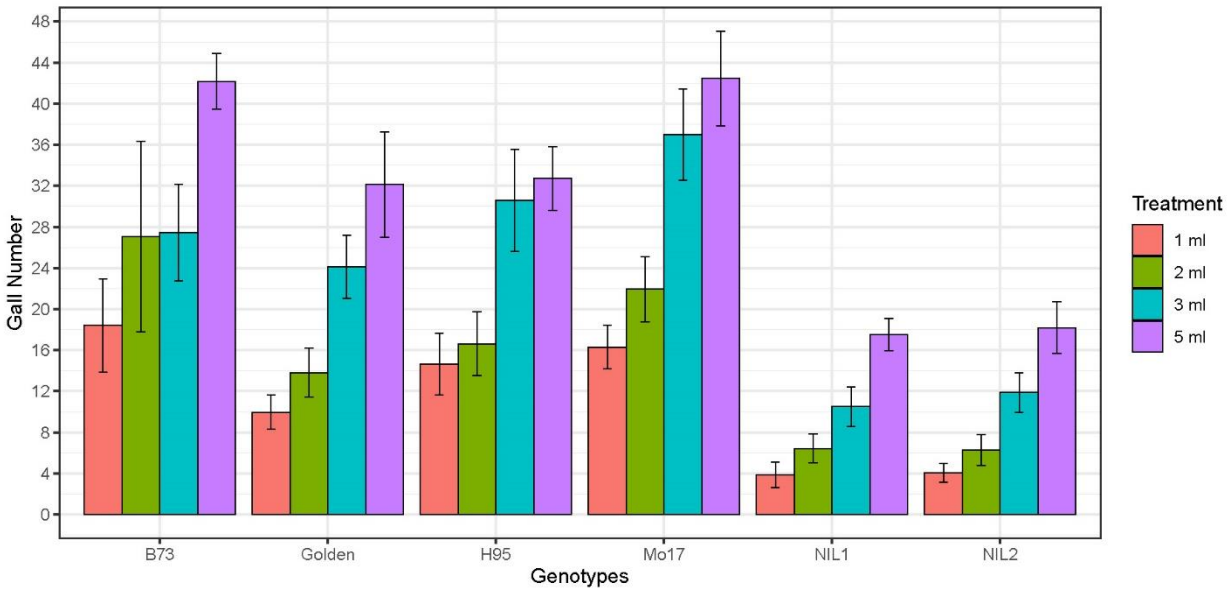

Figure B.2. Mean ear gall number for six genotypes by treatment levels. The x-axis represents four maize genotypes and two NILs, and the y-axis represents the ear gall number. The colored legends indicate the four treatments.

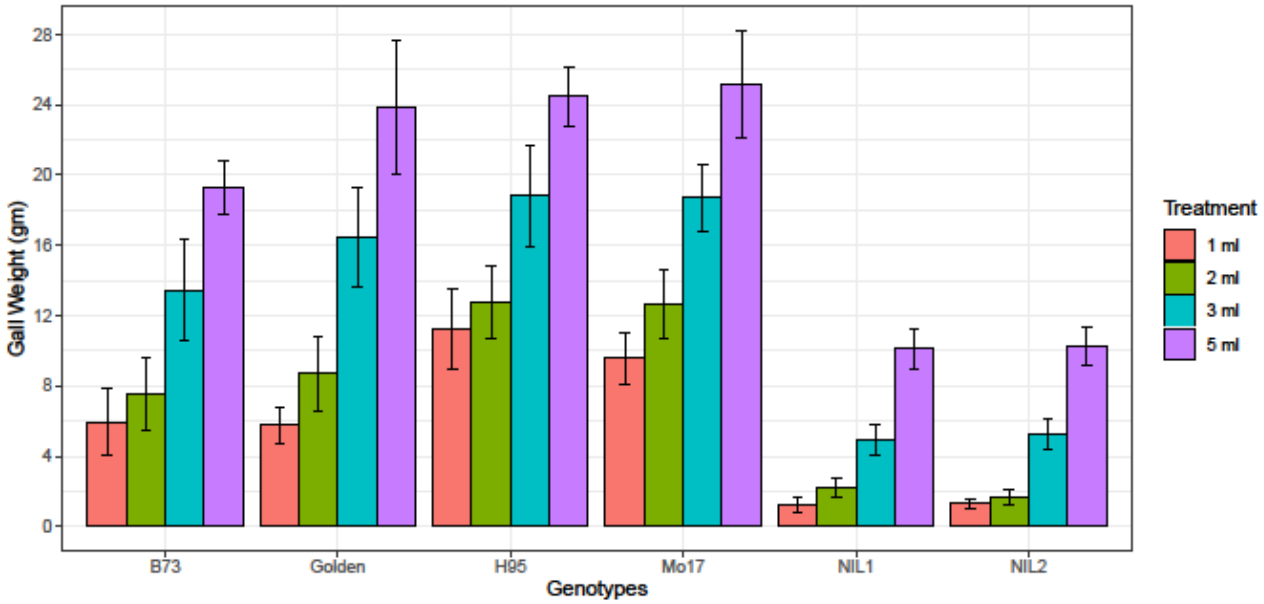

Figure B.3. Mean ear gall weight for six genotypes by treatment levels. The x-axis represents four maize genotypes and two NILs, the y-axis represents ear gall weight (gram) and the colored legends indicate the four treatments.

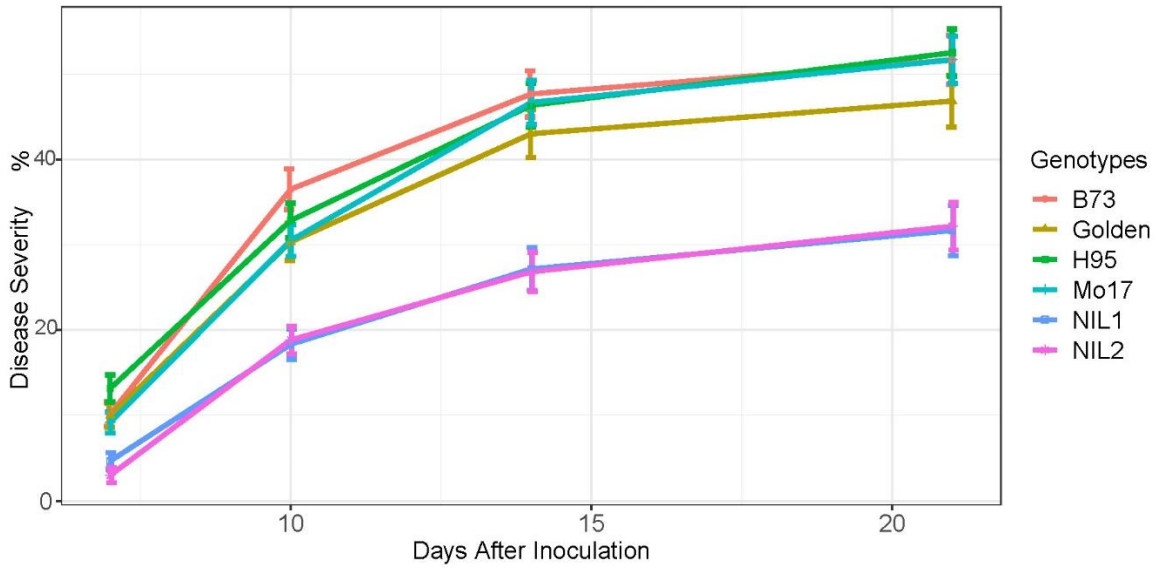

Figure B.4. Ear disease severity for six genotypes at 7, 10, 14, and 21 days post inoculation. The x-axis and y-axis represent days post inoculation and percentage disease severity in ears, respectively. Different colored dotted lines represent six genotypes.

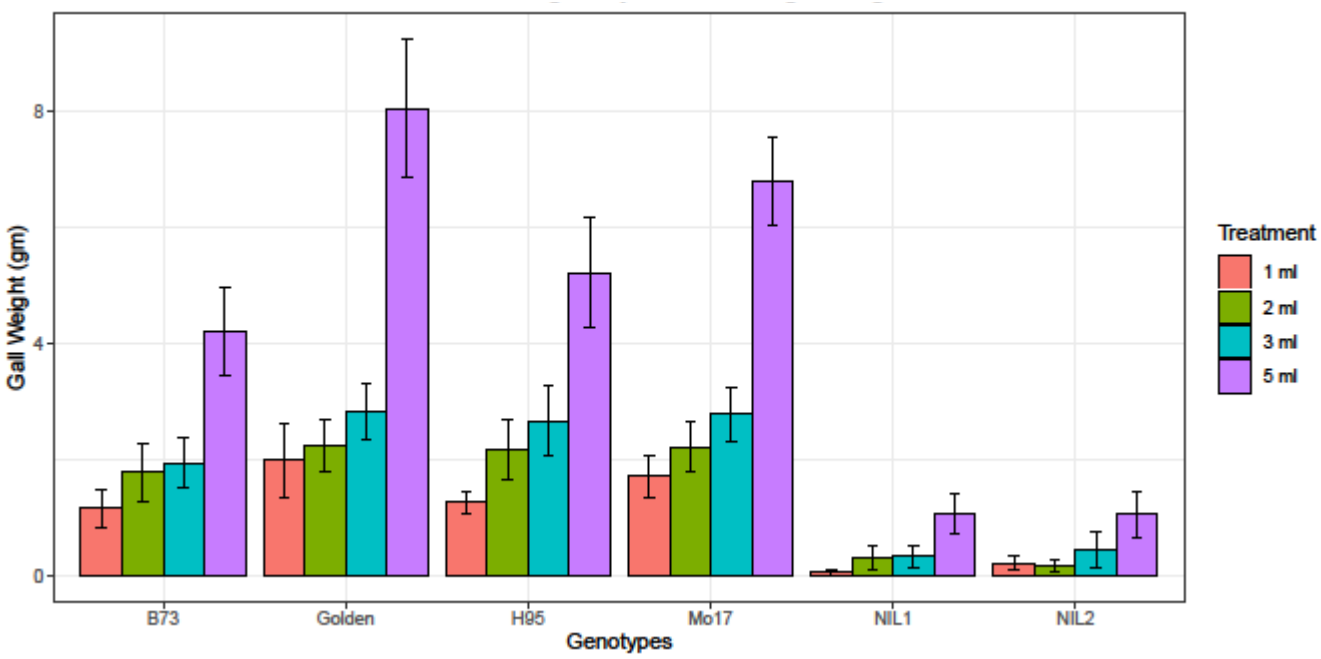

Figure B.5. Mean tassel gall weight for six genotypes by treatment levels. The x-axis represents four maize genotypes and two NILs, and y-axis represents the tassel gall weight in gram, and colored legends represent the four treatments.

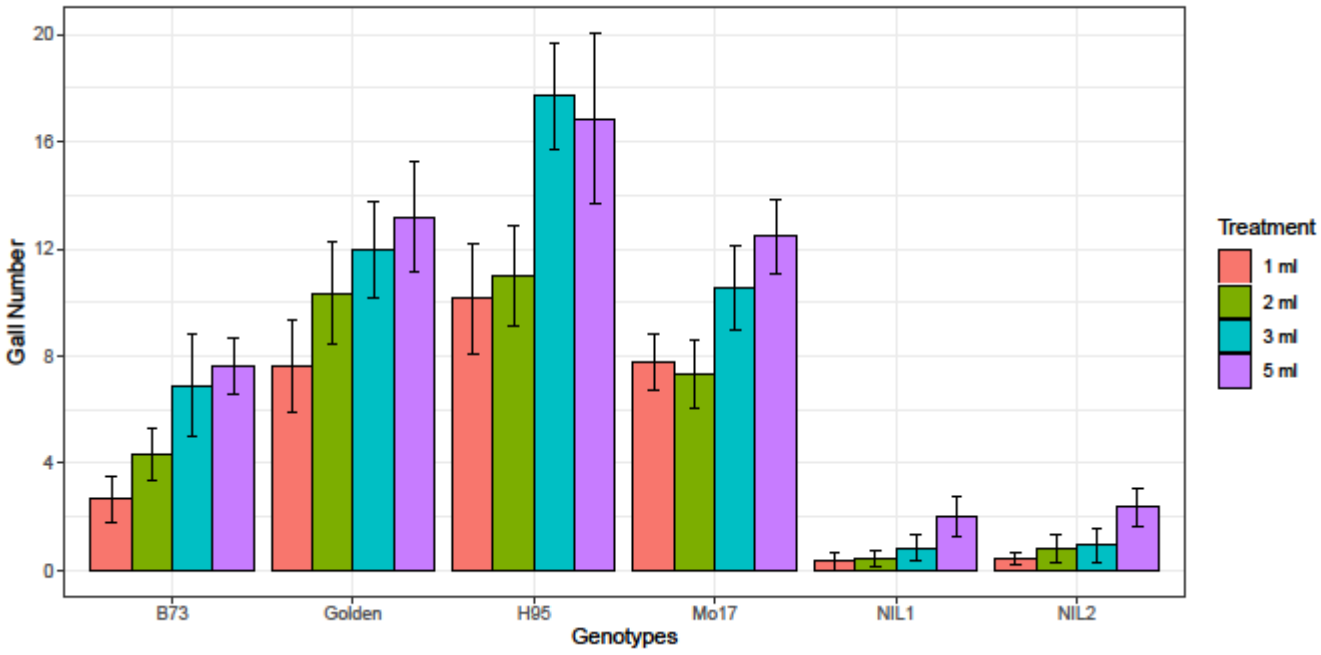

Figure B.6. Mean tassel gall number for six genotypes by treatment levels. The x-axis represents the four maize genotypes and two NILs, and the y-axis represents the tassel gall number.

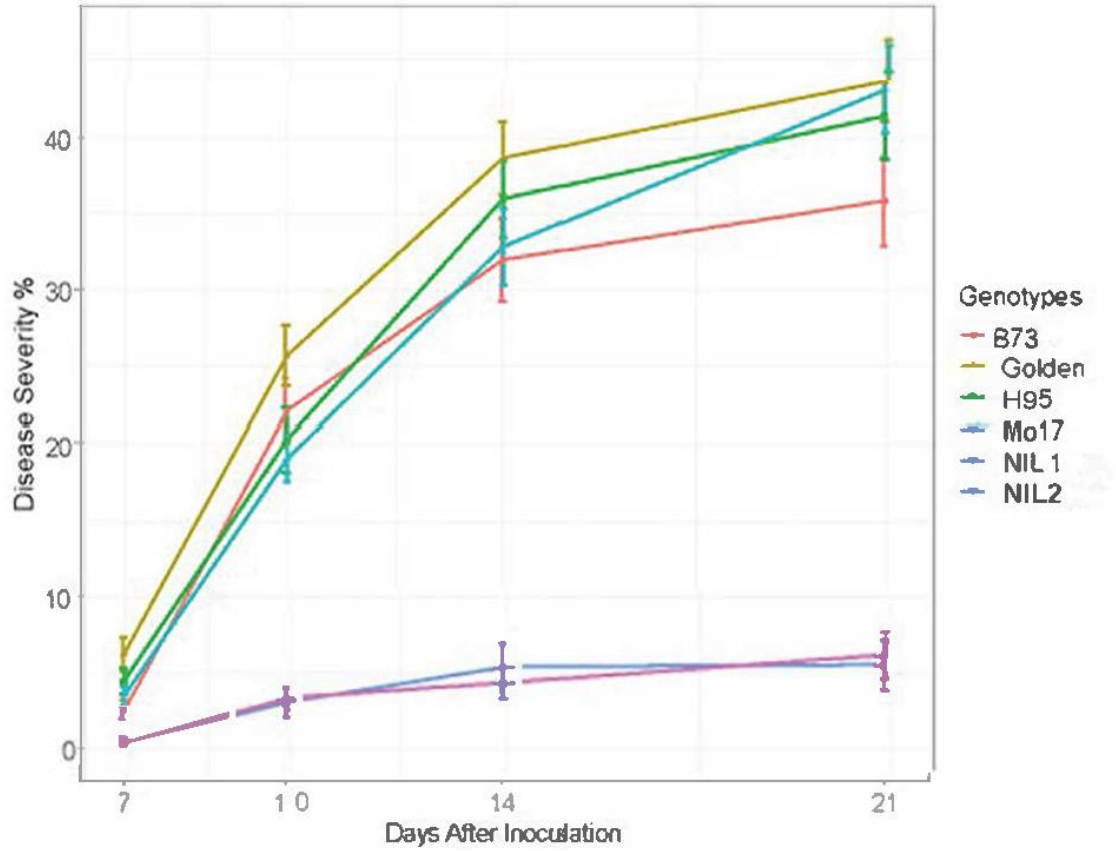

Figure B.7. Tassel disease severity for six genotypes 7-21 days post inoculation. The disease severity was estimated based on visual assessment on a disease rating scale from 0-5. The x-axis represents days post inoculation, and the y-axis represents the percentage of disease severity in the tassel. Different colored dotted lines represent six genotypes.