NEW INSIGHTS FROM THE NORTHERN LEAF SPOT DISEASE OF MAIZE CHALLENGE THE PREVAILING VIEWS OF ADULT PLANT RESISTANCE AND THE NECROTROPHIC MODE OF FUNGAL PARACITISM

by

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ABSTRACT

The Northern Leaf Spot disease of maize is caused by the fungus *Cochliobolus carbonum* race 1 (CCR1), which employs a necrotrophic mode of growth to colonize its host. A key pathogenicity factor that CCR1 employs to invade corn is HC-toxin, which confers on CCR1 the potential to decimate corn of any age. Fortunately, a resistance gene has evolved in maize that prevents CCR1 from causing disease. Named *Hm1*, this resistance gene encodes an HC-toxin inactivating reductase enzyme, HCTR, whose activity is dependent on NADPH as a coenzyme. While the HCTR encoded by the WT *Hm1* protects maize at every stage of development, the HCTRs encoded by the weak, partial loss-of-function alleles of *Hm1* do not. These mutant HCTRs confer disease resistance only in mature plants, and not in young seedlings. The reason is that seedlings are not robust enough metabolically to satisfy the heightened need of the cofactor NADPH for the mutant HCTRs. Genes that confer resistance only in adult tissues are called adult plant resistance (APR) genes, and the resistance that they provide is believed to last for the remainder of the life of the plant after onset. However, our results with APR in the maize-CCR1 pathosystem are at odds with this belief. While maize plants containing the APR alleles turn as resistant as the plants containing the WT allele by anthesis, they gradually become more and more susceptible afterwards during the ear-fill period, and the severity of the disease relating inversely with the HCTR activity of the APR allele. Thus, APR in maize is dictated not by age but by the status of host metabolism.

We also explored the necrotrophic behavior of CCR1 to accomplish host invasion. All necrotrophic pathogens are thought to have the potential to cause disease by taking advantage of any kind of cell death in the host. This includes cell death that accompanies the hypersensitive immune response (HR), as has been witnessed with *Botrytis* and *C. victoriae* in Arabidopsis. Interestingly, this was not the case with CCR1. CCR1 was not able to colonize maize cells dying from HR in an autoimmune mutant, provided the functional Hm1 gene was in the background. These results suggest that not all necrotrophic pathogens are created equal, as has been presumed all along. My results clearly suggest that while some necrotrophs are able to take advantage of the HR cell death for host colonization, others do not.

Another result of key interest is that the HR cell death and the associated induction of the defense response also did not alter the outcome of the susceptible reaction of maize to CCR1. Susceptible plants with the genotype *hm1hm1* were equally susceptible to CCR1 regardless of whether the plants carried the autoimmune gene Rp1-D21 or not. These findings imply that HC-toxin does not induce host susceptibility by interfering with the induction of defense responses, as has been acclaimed.

CHAPTER 1. LITERATURE REVIEW

Plant defense against pathogens

Plants have evolved numerous mechanisms to protect against the invasion of a diverse set of pathogenic organisms (Anderson, 2018). Continuous biotic stress has promoted this evolution to aid in containing pathogens to a small number of cells (Miller, 2017). Multiple factors help distinguish between plant pathogens, but the most prominent is how they acquire nutrition. Necrotrophs feed off of dead or dying tissue while biotrophs feed on living cells to acquire nutrients for growth and reproduction (Agrios, 1997). Biotrophs feed on living tissue to avoid detection, but plants have evolved a programmed cell death known as the hypersensitive response (HR) that undergoes activation at the site of the infection to prevent the spread of disease (Klienbenstein, 2008). It appears that necrotrophs readily use the cell death associated with HR to their advantage. Defense against necrotrophs typically comes in the form of restraining necrosis to prevent the spread of the pathogen (Mengiste, 2011). The ability to defend against pathogens depends upon the type of defense mechanism. These defense mechanisms can be preformed or inducible.

PAMP triggered immunity (PTI) formerly known as basal resistance or basal compatibility is a form of inducible immunity that is called into action upon invasion when physical defenses are defeated (Jones and Dangl, 2006, Johal et al, 1995). In most cases PTI halts infection before the pathogen has the ability to colonize the plant (Shirron, 2011). Pathogens have evolved the ability to suppress PTI by secreting effector proteins into plant cells that ultimately alter the manifestation of resistance responses (Chisholm, 2006); this is known as effector triggered susceptibility. Biotrophs and necrotrophs both have the ability to suppress PTI but the difference is that biotrophs need to keep infected plant cells alive, at least initially (Johal et al., 1995). Biotrophs do so by secreting effectors that suppress PTI but do not compromise the integrity of the host cell (Johal et al, 1995).

Necrotrophs on the other hand have no need to keep host cells alive, the effectors used can be broad functioning to suppress PTI by more radical means (Laluk, 2010). Both specific and

nonspecific toxins are utilized by necrotrophs to induce host susceptibility and debilitate the plant by either slowing down or suppressing the plants defenses (Wang, 2014). Toxin production can be the sole factor in determining the pathogenicity or virulence of an organism (Johal et al., 1995). In the case of *Alternaria alternata* which causes brown spots on citrus leaves and fruits, ACT toxin is utilized by the fungus to cause rapid electrolyte leakage in susceptible cells (Yang, 2012). The contents of these cells are then used by the pathogen to fuel growth and reproduction. This implies that any factor incapacitating host metabolism may render the host more susceptible to necrotrophic pathogens.

Necrotrophs

Necrotrophs unlike most other pathogens extract their nutrients from dead cells that are intentionally killed prior to or during colonization of the host (Mengiste, 2012). The life cycle of necrotrophs generally follows a destructive pathway resulting in necrosis and plant rots (Laluk, 2010). Nutrients are generally obtained with the aid of phytotoxic compounds and sometimes these are host specific. These toxins do so by degrading cell wall enzymes to induce necrosis (Mengiste, 2012). Necrotrophs pursue infection by conidial attachment to the leaf, germination of the conidia, epidermal penetration with the appressorium or through stomatal openings, tissue maceration with cell wall degrading enzymes, then sporulation (Laluk, 2010). The toxins utilized for cell death generally manifest themselves in a way that stops the induction of immune responses that would otherwise mount a defense against the pathogen. Some of these toxins are controlled by a single genetic locus. One example is *Cochliobolus carbonum*, where a single locus *Tox2* controls the production of the host selective toxin HC-Toxin. How a single genetic locus controls the biosynthesis of host selective toxins is not currently known (Walton, 1987). Defense against necrotrophs and their recognition factors and signaling that creates a response to necrotrophic invasion is not well understood and defense against them may vary depending on the primary determinant of virulence (Mengiste, 2021). Selective necrotrophs produce host specific toxins that are generally reduced by resistant plants (Mengiste, 2012).

Adult plant resistance

Nearly all plant disease resistance genes give complete protection at all stages of development in every part of the plant. Other instances exist where resistance is manifested in an age-related fashion. These forms of resistance can confer resistance gradually or at a specific stage of development. The Xa21 resistance gene in rice giving protection against Xanthamonas oryzae pv. oryzae confers very little resistance in the first three weeks but by maturity reaches full efficacy (Song et al, 1995). Several APR genes provide effective protection only at maturity but others like *Yr34* can confer resistance against seedling rust at lower temperatures (Krattinger et al, 2009). Unlike *Yr34, Yr36* confers greater resistance to stripe rust at higher temperatures (Chen, 2013; Fu et al, 2009). No clear evidence explains how changes in the environment affect the developmental regulation of APR genes.

The mechanistic basis of APR is not well understood in any pathosystem but multiple single resistance genes conferring this phenotype have been cloned. Xa21 in rice encodes a receptor-like kinase for protection against Xanthamonas oryzae (Song et al, 1995). The cloning of *Lr34* showed that it confers resistance to multiple fungal pathogens in wheat and codes for a putative ABC transporter (Krattinger et al, 2009). Alleles of *Lr34* were generated by mutagenesis and showed that minor point mutations in *Lr34* resulted in intermediate rust resistance while large changes resulted in a completely susceptible plant. Likewise, cloning of the *Hm2* disease resistance gene in maize revealed that the 52 amino acids of the protein were completely missing at the 3' end when compared to the wildtype *Hm1* allele (Chintamanani et al, 2008). Isolation of *Yr36* in wheat displayed that it contains a kinase and a START lipid-binding domain (Fu et al, 2009). Gene cloning further showed that while several APR genes belong to the class of gene-for-gene, there are other APR genes that do not confer resistance in a gene-for-gene manner. Cloning however did not lead to any real conclusion of the mechanistic basis of APR. Characterizing the transcriptional status of APR genes over different life stages has ruled out differential transcription as the basis of APR in all genes tested (Chintamanani et al, 2008).

Maize-*Cochliobolus carbonum* race 1 pathosystem

Cochliobolus carbonum previously known by its anamorph *Bipolaris zeicola* or *Helminthosporium carbonum* is the causal agent of Northern Leaf Spot in maize. *Cochliobolus carbonum* Race 1 (CCR1) is a devastating pathogen that can infect all organs of the plant at all developmental stages with typical symptoms being leaf blight, ear mold and stalk rot (Ullstrup, 1941; Sindhu et al., 2008). Fortunately, most of the maize germplasm is resistant to this pathogen, preventing it from causing economic loss to the maize crop. CCR1 utilizes the host selective toxin (HC-toxin) as the causal agent of its pathogenicity (Scheffer and Ullstrup, 1965). Race 1 of *C. carbonum* produces HC-toxin while race 2 does not. The role of HC-toxin was proved by exogenously applying it to the site of infection in the presence of race 2, where it then produced disease lesions similar to that of race 1 (Sindhu, 2008). The application of HC-toxin after inoculation with race 2 has shown that the toxin shuts down the expression of defense genes (Young, 2008). From this it can be theorized that HC-toxin interferes with JA/ET mediated defense responses in the host plant to promote susceptibility.

Complete resistance in maize at all developmental stages to CCR1 is conferred by a single copy of the resistance gene *Hm1*. Transposon mutagenesis of *Hm1* divulged its ability to encode HC-toxin reductase (HCTR), an NADPH-dependent HC-toxin inactivating enzyme (Johal and Briggs, 1992). HCTR functions to reduce HC-toxin and gives Hm1 its complete protection against CCR1 (Meeley et al., 1992). Multiple variants of Hm1 exist that confer immunity in a developmental fashion; this includes a duplicate of hm1 at the syntenic locus hm2 (the wild type *Hm2* allele). Various instances of APR have been found in several pathosystems, but despite the isolation of multiple APR genes, involved in both R and non-R type resistance, APR remains unexplained (Marla, 2018).

In the maize CCR1 pathosystem, the transcriptional levels of *Hm2* and *Hm1A* have been studied and they do not appear to change with the increase in age and growth (Marla, 2014). *Hm1A* was cloned and the HCTR encoded differs from the WT *Hm1* by 5 amino acids (Marls, 2018). New APR alleles were generated from *Hm1* by targeted EMS mutagenesis which involve single point mutations changing G/C into A/T. 7 alleles were generated, 2 displaying APR and 5 susceptible

throughout. Sequence analysis of these mutant allele showed that while the 2 APR alleles carried missense mutations, the 5 completely susceptible *hm1* alleles had undergone nonsense mutations.

The underlying cause why partial loss-of-function mutations of Hm1 cause APR is still not completely understood. Differential transcription and translation have been ruled out for the cause of APR. Two theories have arisen based on these conclusions; one possibility is that there are other factors that are increasing the HCTR activity of APR genes. The other possibility is that posttranslational changes in each HCTR is increasing their activity. Since HCTR requires NADPH to reduce HC-toxin, this leads us to believe that the metabolic status of the plant is somehow responsible for the APR phenotype. When maize plants carrying Hm1 were exposed to conditions that altered their photosynthetic output a decrease in resistance to CCR1 was observed (Marla, 2018). Placing maize plants in the dark completely deteriorates their resistance to CCR1. Maize plants grown in the greenhouse during the wintertime show weaker resistance to CCR1 as opposed to plants grown in full sun in the field

Host-pathogen energetics

Strict regulation of plant defenses can be critical to the plants health because defense responses require a large quantity of energy and when left unchecked can use valuable energy that otherwise would aid in reproduction and growth (Stamp, 2003). The ability to reduce and or stop plant defenses to the site and time of infection could be the difference between the plant dying before maturity and reproduction (Stamp, 2003). Both PTI and ETI include reprogramming of the host cells at the site of attempted infection. Primary metabolism has been thought to be the determining factor because it supports cellular energy requirements for various plant defense responses (Bolton, 2009). What happens metabolically when reprogramming of infected cells is compromised by conditions of stress? The role of metabolism in plant defense has been given little attention over the years, though it has been recognized as an important factor in plant disease (Rojas, 2014).

Disease Lesion Mimic Mutants

Disease lesion mimic mutants in plants confer spontaneous disease-like symptoms in the absence of any inflicted injury by pathogens, or mechanical damage (Johal, 2007). Lesion mimic plants

can have various complications not only related to disease responses. The defects in lesion mimic plants allow for the study of defense responses which are typically upregulated at the time of pathogen invasion (Johal, 2007). Resistance genes are deployed to defend against the colonization of pathogens, these resistance genes (R genes) recognize secreted molecules from the pathogen known as effectors (Sun et al., 2020). A plants ability to recognize effectors from pathogens allows it to trigger a series of highly regulated defense mechanisms (Freeman 2008). One specific mechanism of interest is the rapid localized cell death known as the hypersensitive response (HR). HR is used to contain a pathogen from spreading by restricting it to the site of infection (Balint-Kurti, 2019). However, HR is detrimental to plant growth and development if it is triggered spontaneously or not contained properly (Balant-Kurti, 2019). Mutants that trigger HR inappropriately are called autoimmune mutants. Such mutants arise from mutations within an R gene or in host proteins that keep R genes under check in the absence of infection (Balant-Kurti, 2019). Some of the first examples of R gene mutants conferring spontaneous HR came from the maize Rp1 locus, which confers resistance to common rust caused by the pathogen *Puccinia sorghii* (Pataky, 2001). Rp1 is a complex locus containing multiple R-gene analogs (RGAs). The autoimmune mutant Rp1-D21 was produced as a result of unequal crossing over at the Rp1 locus, followed by recombination between two RGAs that resulted in a chimeric gene that confers an autoactive lesion mimic phenotype in the form of HR (Chintamanani, 2010). The Rp1-D21 protein causes spontaneous activation and formation of HR due to the fact that recognition and elicitation functions are uncoupled (Olukolu, 2014). In *Rp1-D21,* HR lesions spontaneously form all over the plant and are profoundly affected by environment, developmental stage, and genetic background (Olukolu, 2014). Lesion formation is uniform in the greenhouse and field settings with gradual progression up the plant as it ages. HR is generally associated with pathogen resistance but in some specific circumstances it can hinder the growth of plants and increase pathogen susceptibility. Plants implement multiple mechanisms to suppress such plant defense responses as well as constraining it after its activation (Freeman, 2008). Severe forms of the autoimmune mutant *Rp1-D21* cannot turn off its extensive HR response and this ultimately leads to the demise of the plant prior to reproduction (Pataky, 2001).

To understand the resistance response associated with HR, there is a need to have it turned on in the absence of a pathogen. *Rp1-D21* lacks the ability to suppress HR in the absence of a pathogen

allowing for the study of HR without the adverse effects of a pathogen. Learning more about HR can improve our understanding of how it works against biotrophs and not against necrotrophs. Autoactivate immune mutants present a key tool to help us study the interaction between HR and necrotrophic pathogens.

Research Objectives

There were two objectives of my thesis research. The first was to monitor how APR conferred by different alleles of Hm1 progresses over time in relation to plant growth and development. Disease ratings were taken at weekly intervals not only up to the flowering stage but also following pollination. The rationale was that the maize plant goes through different phases of source-sink changes that impact the allocation of photosynthates. Marla et al. (2018) showed that the key driver of APR in the maize-CCR1 pathosystem was the metabolic robustness, which they argued largely lacked in the seedling tissues because of their limited photosynthetic capacity. It is also well known that major changes in source allocation happen after pollination during the ear-fill period. And if the plant is unable to fully satisfy the sink capacity of developing ears, tissues that are relatively dispensable are cannibalized to recycle Carbon and Nitrogen into developing kernels. One consequence of this allocation reprogramming is the compromised metabolic status of the lower leaves on the plant. If what Marla et al (2018) found is true, then we expect APR to erode in these leaves as the plant ages during the ear-fill period.

The second objective was to look into the necrotrophic mode parasitism of CCR1, which is considered a necrotrophic pathogen requiring dead or dying cells for infection. Research has been published that claims that any form of host cell death has the potential to facilitate necrotrophic invasion. This includes the cell death that is often associated with the hypersensitive response, one of the plant kingdom's most effective immune response. Can CCR1 also manipulate HR cell death to establish infection of maize? This question was asked because CCR1 was never reported to be associated with lesion mimic mutants, many of which exist in maize, and they all involve spontaneous cell death. As discussed earlier, a few of these have been shown to undergo HR cell death as a mechanism of lesion development. One HR cell death-involving lesion mimic mutant that has been characterized in detail in the Johal lab is *Rp1-D21*.

To address if HR cell death has the potential to make maize susceptible to CCR1 in any way, the *Rp1-D21* autoimmune gene was introgressed into B73 containing and lacking the *Hm1* gene.

CHAPTER 2: APR IN THE MAIZE-COCHLIOBOLUS CARBONIM RACE 1 PATHOSYSTEM IS DICTATED NOT BY THE AGE OF THE HOST BUT BY THE STATUS OF ITS METABOLISM

Abstract

APR is a phenomenon in which plants are susceptible to disease as seedlings but become resistant at maturity. Implicit in this description is the belief that once manifested, APR lasts for the life of the plant. However, some recent research from our lab on APR using the maize-CCR1 pathosystem suggested that it is the metabolic vigor of the host that determines APR largely. Maize resistance that displays an APR phenotype is conferred by partially mutant alleles of *Hm1* that encode weak HCTRs. These mutant HCTRs require much higher levels of the cofactor NADPH than the wild-type HCTR, and maize seedlings are not robust enough metabolically to meet this need. If this is true, APR should also fail at other times in the life of the plant when their metabolism gets compromised. One such time in the life of the maize plant coincides with the ear fill period when the plant undergoes a major reprogramming in resource allocation. To address if changes in energy allocation towards the developing ear reduces the plants' ability to resist CCR1, disease symptoms were measured on plants containing and lacking APR alleles, before and after fertilization on a weekly basis. As expected, full resistance was achieved at maturity by plants containing *Hm1* alleles, regardless of their strength. However, shortly after pollination plants containing the APR alleles began to display a decrease in resistance. This decrease was most obvious on plants containing the weaker APR alleles, so much so that their foliage was largely blighted by week 7 after pollination. In contrast, the foliage on plants containing the WT allele was still green at this time with no signs of CCR1 or NLS symptoms. These findings suggest that APR is in fact dependent on the metabolic status of the plant, and not dictated simply by the age of the plant.

Introduction

Plants have evolved multiple responses to pathogen invasion that involve a number of inducible mechanisms. Proper timing for activation is critical to successful prevention of pathogen invasion so these mechanisms are only initiated when infection is sensed. The detection of pathogens is critical to defense due to their costly energy consumption if activated at the wrong time. Most

genes that confer resistance do so at every stage of growth. However, plants also exhibit stage specific or organ specific resistance. Adult plant resistance (APR) is a phenomenon in which plants are susceptible to disease as seedlings but are resistant at maturity. Other terms, such as age associated resistance, ontogenic resistance, and mature plant resistance, have been used in the literature to describe the same phenomenon. Three different alleles of a resistance gene present at the hm1 locus display the APR phenotype. In most cases APR gets stronger with plant age, but in some instances, the onset of resistance happens quickly and at a specific stage of development. The resistance given by APR may not be robust at the seedling stage but APR confers resistance that is unlikely to be lost as disease pressure increases. Most pathogens contain significant variation for avirulence/virulence to specific resistance genes. Thus, promoting boom and bust cycles of disease resistance to be common in modern agricultural fields because many R genes lose their ability to confer resistance when the corresponding Avr gene in the pathogen mutates, the Avr product can no longer be recognized by the R gene protein (Jones and Dangl, 2006). When this happens, R genes lose their effectiveness, and this leads to reduced resistance and yield in subsequent seasons. APR genes can add robust resistance that is not subject to simple mutations in the pathogen (GRDC, 2012). Most resistance is conferred by one singular gene making them vulnerable to a pathogen because the pathogen only has to mutate once to overcome it. APR leaves the seedling vulnerable but resistance at the adult stage is robust and like Hm1 has prevailed as with using multiple gene pyramiding, the addition of alleles conferring APR along with other R genes can help keep important R genes effective by adding more resistance for the pathogen to overcome. APR genes such as Lr34 and Lr67 confer broad spectrum partial resistance against multiple pathogen species in this case they provide resistance to several mildew and rust pathogens (Soria, 2019). Most APR genes confer weak resistance to detrimental pathogens but when one line of wheat contains 4-5 APR genes they can act additively and display a high level of resistance (Singh, 2010).

Cochliobolus carbonum (CCR) causes northern leaf spot and it is an excellent model system because *C. carbonum* relies entirely on a single toxin, HC-toxin, that is produced by the pathogen and that is chemically reduced in the plant (Marla, 2018). It is not understood why APR alleles confer late resistance, even though a large number of genes that result in APR in a number of species have been cloned and characterized. The dominant allele of *Hm1* confers complete

resistance throughout the life cycle of maize. This *Hm1* allele encodes a protein with 356 amino acids. In contrast, the protein encoded by the syntenic gene, *Hm2* is truncated, lacking 52 amino acids when compared to *Hm1* (Dehury, 2014). This truncation results in a protein that confers a weak APR phenotype. Multiple new APR alleles of Hm1 have been generated via EMS seed mutagenesis of resistant *B73* carrying *Hm1*. Analysis of these alleles helped to confirm a causal relationship between the weak nature of APR alleles and their phenotypes. Truncation in these new APR alleles displays that APR is a consequence of mutations in *Hm1* that causes a partial loss of function, resulting in seedling susceptibility. We hypothesize that HC-toxin reduction is the key variable because the level of accumulated gene product and the degree of gene expression in seedlings carrying APR do not match the reduced resistance exhibited by these alleles. The quantity of toxin that is reduced by APR alleles does not increase over time as the plant matures. This displays that the plant has the ability to reduce the toxin but does not have the key NADPH to drive enough HC-toxin reduction at the seedling stage. With no change in toxin reduction from seedling to adult stage in *Hm1* plants and APR alleles there must be a change in the availability of usable NADPH to reduce the toxin. This suggests that HC-toxin reductases encoded by the APR alleles require more NADPH to reduce the same amount of toxin (Chu, 2014). As plants mature and leaf size increases, photosynthesis increases, allowing for a surplus of NADPH to be used on resistance (Dwyer, 2003). Many lines of evidence suggest that the disruption of metabolism has an impact on disease resistance in maize. A reduction in photosynthesis due to decreased light exposure decreases resistance conferred by both APR alleles and in fully resistant plants carrying *Hm1* (Marla, 2018). Maize grown in the greenhouse exhibits a large reduction in resistance relative to field grown maize, and plants closer to supplemental lighting have a greater resistance response than those distant from light sources. APR alleles in a double mutant combination with dominant oil-yellow-N1989, which has a chlorophyll deficiency, reduces its resistance to pathogens (Marla, 2018). Weak *Hm1* alleles confer resistance when taken from 12 hours of light to 18 hours, demonstrating that an increase in available photosynthetic metabolites can enhance the resistance response. Further, exposure to the herbicide DCMU, which disrupts electron transfer during the light reactions of photosynthesis, also reduces resistance. NADPH and NADP+ quantified samples taken from juvenile (V3) and mature (V12) leaves showed that during the day mature plants contained a significantly larger amount of both NADPH and NADP+ (Chu, 2014). Chu et al displayed that even at saturated levels of NADPH there was not enough to increase

HCTR activity in APR alleles. No change in toxin reduction overtime and with NADPH being the limiting variable we hypothesize that APR alleles require more NADPH to reduce enough toxin to produce a resistance phenotype than do dominant wild type alleles. Grain fill after fertilization in plants triggers the production of kernels that are considered to be large sinks that uptake a majority of the plants nutrients during this stage (Borghi, 2017). The consumption of photosynthetic metabolites for grain fill may lead to a reduction in the available NADPH that can be used to reduce HC-toxin. During the fill period maize begins to cannibalize lower leaves that have a lower photosynthetic production to conserve resources. This remobilization of nutrients can cause an increase in susceptibility in the lower leaves and roots (Nielsen, 2003). This lead us the hypothesis that APR is caused by a change in metabolism in plants at the seedling stage relative to plants undergoing mature growth.

With NAPDH being the primary limiting factor that is required for HC-toxin reduction in maize, we hypothesize that the process of producing an ear will be a large enough sink to affect the resistance seen in APR alleles. If the plant allocates NADPH to the production of an ear, we expect to see a reduction in resistance after fertilization due to the loss of NADPH used to support HCTR. This hypothesis was addressed by collecting data prior to fertilization and comparing it to data collected post fertilization to determine if any change in resistance is associated with the grain fill period. Here, with lesion data we confirm that APR alleles do in fact display a decrease in resistance after fertilization.

Materials and Methods

Inoculation and plant material

The protocol used to make carrot agar juice medium and culturing CCR1 is the same that was previously described in (Johal and Briggs, 1992). Three-hundred µl of 50,000 spores/mL of CCR1 conidial suspension was used for leaf whorl inoculations. To document the phenotypic manifestation of APR we planted all APR alleles currently studied in our lab. Resistant B73 was used in both homozygous (*Hm1Hm1*) and heterozygous (*Hm1hm1*) conditions to show that only one copy of *Hm1* is necessary for complete resistance and were used as the resistant controls. *Hm1-2* (*hm1hm1*) was used as the susceptible control and was created via EMS mutagenesis. Alleles

found at the *Hm1* locus: (*Hm1-3, Hm1-4, Hm1^a*) along with the only allele found at the *Hm2* locus (*Hm2*) along with the heterozygous condition for each were used to display phenotypic manifestation of APR. All material was planted via six replications in adjacent rows, each containing sixteen seeds for a total of ninety-six seeds per block.

Plant rating and data collection

All plants were planted at Purdue ACRE farm in two separate fields and the first two rows of each block were inoculated with 300 µl of 5^4 spores/mL pipetted directly into the whorl of three-week-old plants. Initial ratings were taken 3 days post inoculation (dpi) then weekly to capture APR. Disease severity ratings were taken on a scale from one to ten. One being completely resistant and ten being completely susceptible. Resistant *Hm1* was consistently given a score of one throughout the growing season and susceptible *hm1* was consistently given a ten. Plants were rated on initial disease symptoms then on a whole plant basis. Six plants were rated and an average was taken from the sum of all six plants. Plants were taken to maturity and allowed to fertilize. Disease ratings were taken from week ten to week 14 post fertilization. A one way ANOVA was used to denote significance between each datapoint within each allele.

Surface sterilizations

One month after whorl inoculation samples were taken from three sections of the plant. Leaf discs were taken to determine the presence of CCR1 on the plant. Leaf discs were taken with a Harris Uni-core sampling tool and placed into 1.5 mL centrifuge tubes. Ten percent bleach was added to each tube for ten minutes to completely sterilize the surface of each leaf. Each leaf disc was washed three times in ddH20 to clean off any remaining bleach. Leaf discs were dried and allowed to culture on carrot agar juice medium as described in (Johal and Briggs, 1992).

Results

Phenotypic manifestation of APR alleles pre-fertilization

While Marla et al. (2018) clearly demonstrated the strength and progression of the all current alleles of APR and its syntelog *Hm2* we used the time before fertilization to characterize the alleles behavior to help realistically capture the changes in resistance from the time after fertilization to harvest. Figure 1. A displays that *Hm1-3, Hm1A, Hm2, and Hm1-4* showed little to no control over the disease at 3 weeks after planting but showed a steady decrease in lesion rating by week 7. At week 7 all alleles had dropped to a rating of 5 or less. Among the APR alleles *Hm1-3* displayed the strongest resistance response to CCR1 ending at 1.5 and *Hm1-4* conferred the weakest response at 4.5. By week 7 all APR phenotypes reached a resistant phenotype that allowed them to produce reproductive structures and undergo fertilization. These results displayed a clear APR phenotype in all APR alleles and the syntelog *Hm2* and helped us gain data pre-fertilization that could help us determine any change in resistance post-fertilization.

Phenotypic manifestation of APR alleles post-fertilization

Fertilization was initiated at or around 8 weeks after planting and continued for two weeks until all plants were fertilized and tassels begun to senesce. Once fertilization was complete disease ratings were taken each week for five weeks 10, 11, 12, 13, 14. Over the course of the 5 weeks post-fertilization all alleles of *Hm1* and its syntelog *Hm2* displayed a decrease in resistance. *Hm1-4* saw the largest decrease in resistance response after fertilization reaching a disease rating of 9 at the culmination of the experiment. *Hm1-3* reached a disease rating of 5 a change of 3.5. Comparing the results from pre-fertilization to the post-fertilization results, displays that the alleles of *Hm1* and its syntelog *Hm2* lose resistance after fertilization during the grain fill period. From this it can be inferred that APR is not a one-way street to resistance and that the resistance in APR alleles may be connected to the current state of host metabolism. These ratings indicate that there is something that changes in host metabolism that corresponds with fertilization and the grain fill period.

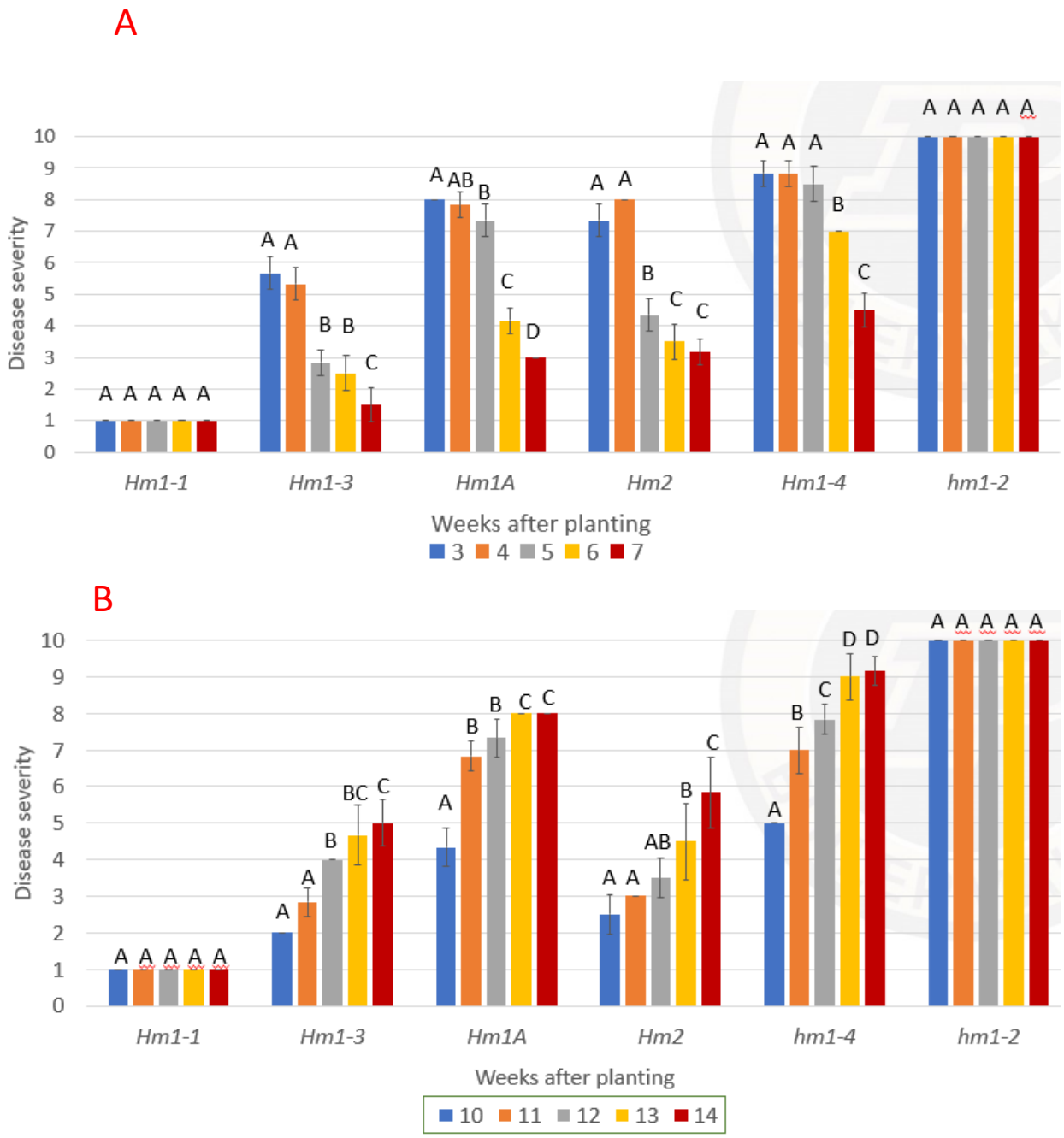

Figure 1. (A). Disease ratings of *Hm1-1* (resistant), *Hm1-3, Hm1A, Hm2, Hm1-4, and hm1-2* (susceptible) plants at weeks 3, 4, 5, 6, 7 after planting. (B). Disease ratings of *Hm1-1* (resistant), *Hm1-3, Hm1A, Hm2, Hm1-4, and hm1-2* (susceptible) plants at weeks 10, 11, 12, 13, 14 after planting.

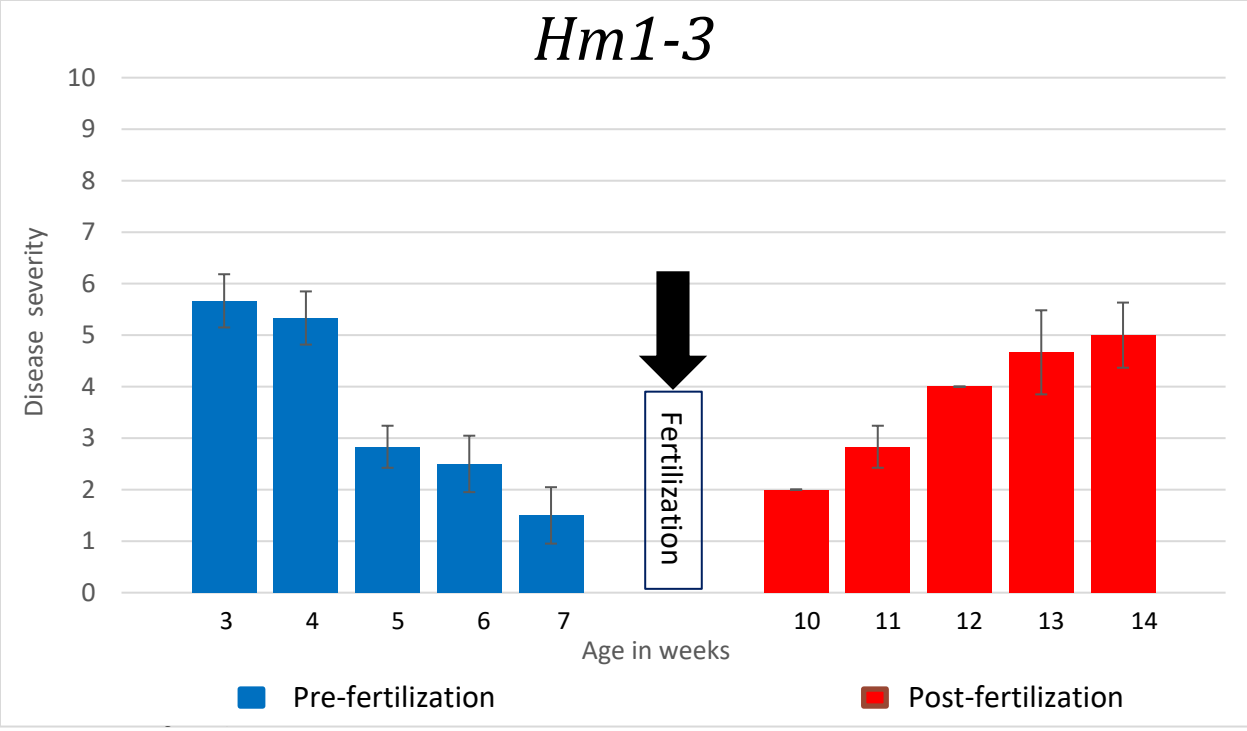

Figure 2. Dynamics of the increase and decrease of resistance mediated by the *Hm1-3* APR allele during the pre- and post-fertilization stages of plant development, respectively.

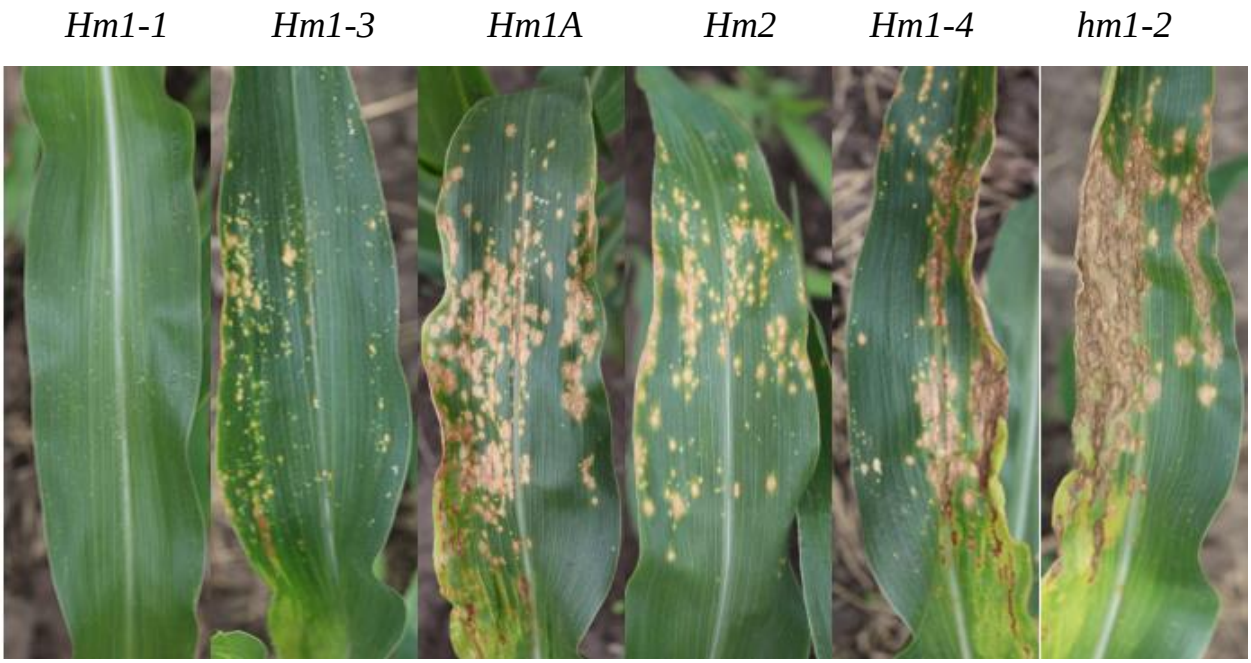

Figure 3. Resistance response at 3 weeks after planting. APR alleles at 3 weeks display a susceptible phenotype when inoculated with CCR1. *Hm1* and *hm1* were the resistant and susceptible controls.

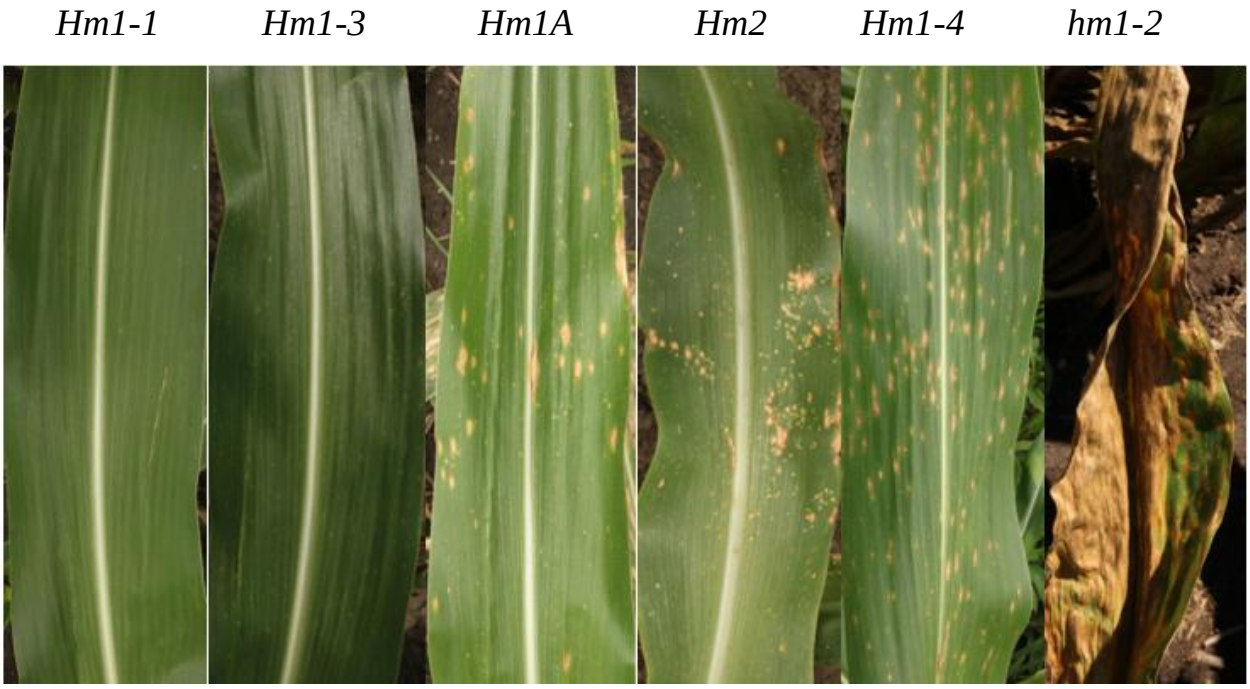

Figure 4. Resistance response at 7 weeks after planting. APR alleles at 7 weeks after planting all turning resistant with age. *Hm1 and hm1* were the resistant and susceptible controls

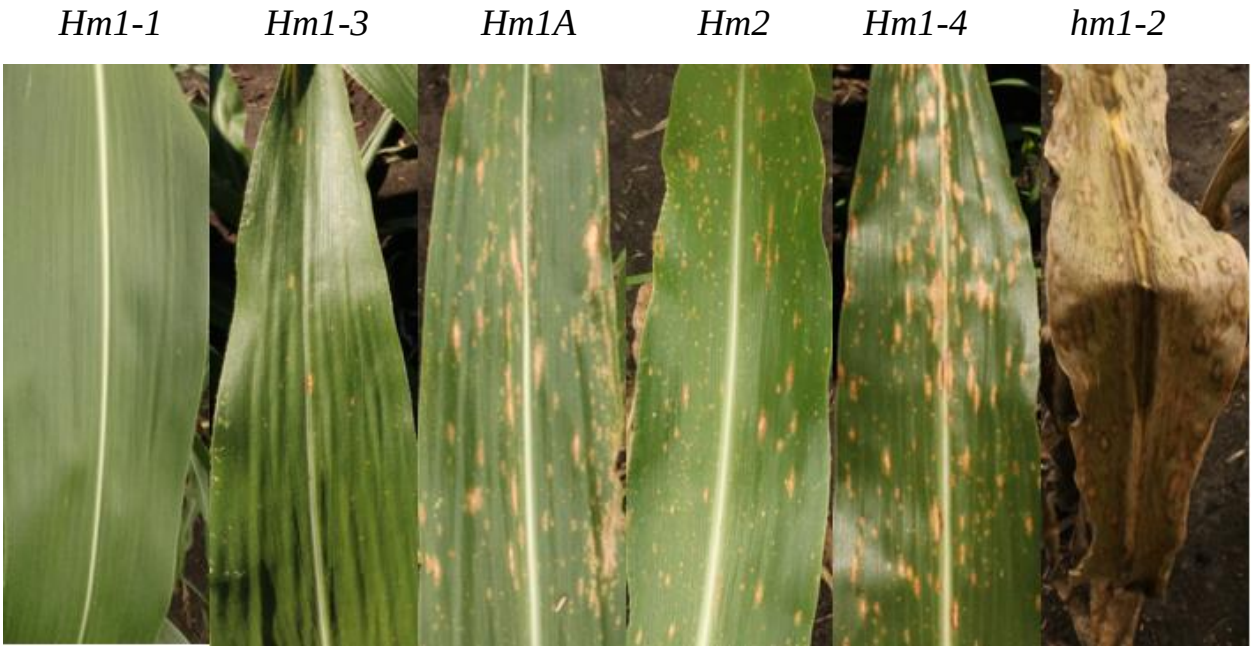

Figure 5. Resistance response 10 weeks after planting.

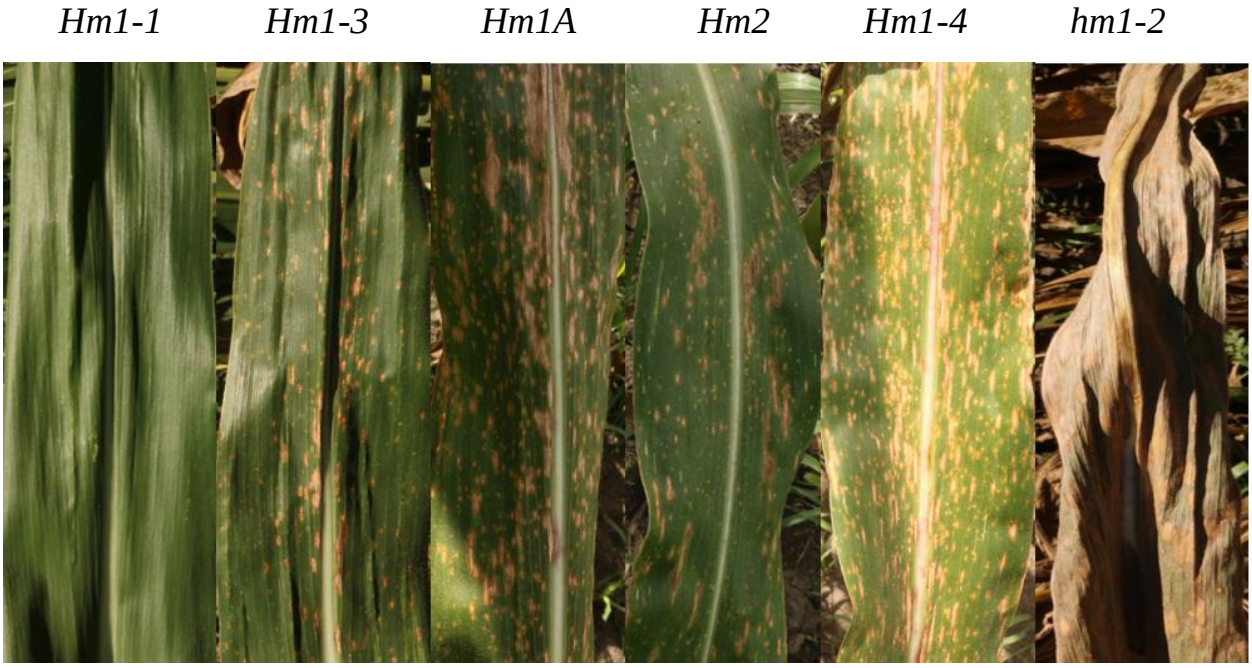

Figure 6. Resistance response 14 weeks after planting.

Discussion

The underlying molecular mechanisms of APR have not been completely characterized though several genes displaying APR have been cloned. Each pathosystem presents a unique challenge when it comes to defining the molecular basis of APR. The maize-CCR1 pathosystem presents a potential link between host metabolism and immunity. While this study was able to determine that APR is not a one-way street giving resistance to CCR1, it does not determine the underlying molecular mechanisms that produce the reduction in resistance after fertilization. These results do provide more insight on the possible link between the metabolic state of maize and its ability to resist against CCR1 and the role of NADPH/NADP+ in resistance to CCR1. The results produced here have helped determine that more work needs to be done on quantifying NADPH/NADP+ metabolites in the plant, specifically before and after fertilization.

NADP(H) metabolites play a fundamental role in the creation of reactive oxygen species (ROS) and the reduction of HC-toxin. Chu et al. showed that NADPH and NADP+ levels are higher in adult plant tissues during the day than in juvenile tissue. The explanation may be that older plants have the ability to create more NADP(H) metabolites and store them due to increased photosynthesis production allowing them to utilize these metabolites to reduce the harmful ROS-by products. Mature plants begin to cannibalize lower leaves due to an increased demand for photosynthetic metabolites during the grain filling period. This remobilization of photosynthates also happens in the lower stalk and root tissues. It can cause deterioration of the lower stalk and can reduce resistance to root and stalk rotting organisms (Nielsen, 2003). With such a large demand for photosynthates during the grain fill period we can infer that similar metabolites being used for resistance are being allocated towards grain filling. During the grain fill period when the bottom leaves are beginning to cannibalize we observed that disease begins from the bottom of the plant and starts to work its way up the plant as time increases. Similar to this we hypothesized that juvenile plants lack the availability of NADPH and NADP+ to reduce enough toxin to produce a resistance response. Maturity brings an excess of NADP(H) metabolites to successfully reduce a similar amount of toxin as the resistant wild type. As plants mature and enter the grain fill period, NADP(H) metabolites may be allocated towards the grain fill period which takes away from resistance. We determined that APR plants lose their resistance gained at maturity after they undergo fertilization. Disease ratings showed that the weakest allele *Hm1-4* nearly reaches the

disease severity of the fully susceptible *hm1-2*, 3 weeks after fertilization. These results tell us that there is something that happens during the grain fill period that has the ability to reduce maize's resistance to CCR1 in APR alleles. The sink source relationship between the plant and ear during the grain fill period seemingly takes away enough photosynthetic metabolites from resistance mechanisms to produce a susceptible response. The resistant control *Hm1-1* did not show a change in resistance after the grain fill period. This further strengthens our argument that the HCTR's deployed by APR alleles require more NADPH than their counter parts deployed by *Hm1*. From this data we can infer that there is likely a connection between host physiology and disease resistance. During fertilization some species drastically shift their energy reserves towards grain fill (Bazzaz, 2005). This could potentially mean that maize allocates its photosynthetic metabolites towards grain fill leading to a lack of available metabolites such as NADPH and NADP+ that can be allocated towards resistance. The lack of NADPH and NADP+ available for HCTR's in APR alleles that require more NADPH to reduce the same amount of toxin could potentially be the reason we see a reduction in resistance after fertilization. Maize is known for its annual life cycle, where it grows to maturity sets seed and senesces shortly after. With this in mind we can infer that maize is potentially using all of its NADPH and NADP+ to produce an ear because it is not conserving energy to over winter like perennials. This information can tell us that NADPH and NADP+ availability and usage in maize could be the determining factor for the gain and loss of resistance in APR alleles. The resistant control *Hm1-1* does not require more NADPH and NADP+ to reduce enough toxin to confer resistance thus when more NADPH and NADP+ are allocated to the ear during the grain fill period it does not undergo a reduction in resistance. From this study and previous studies providing evidence that any interruption in maize's flow of photosynthetic metabolites causes a reduction in resistance we can infer that there is a link between host metabolism and resistance to pathogens.

During the course of our study we determined the change in resistance during pre and post fertilization. This displayed that the alleles of *Hm1* lose the resistance gained from the juvenile stage to maturity. Chu et al. determined that there is a difference in NADPH and NADP+ levels between juvenile and mature plants. Further characterization of pre and post fertilization levels of NADPH and NADP+ will help determine if there is a shift in the total of these metabolites. It is also critical to determine the levels of NADPH and NADP+ in source and sink tissues of maize.

Measuring NADPH and NADP+ in the stalk ear could decipher a change in resource allocation towards the ear during the grain fill period. These results showing a difference in pre and post fertilization resistance in APR alleles have helped us determine that further investigation could potentially provide the answer to the underlying mechanisms of APR. Determining the difference in the NADPH and NADP+ pool before and after fertilization could help further determine the role of NADPH and NADP+ in HCTR and ultimately the reason for APR.

CHAPTER 3: THE INABILITY OF CCR1 TO EXPLOIT THE HYPERSENSITIVE RESPONSE (HR) CELL DEATH FOR NECROTROPHIC COLONIZATION OF ITS HOST

Abstract

CCR1, the causal agent of NLS and whose virulence is mediated by a host-specific toxin (HC-toxin), is considered a necrotrophic pathogen. Pathogens using the necrotrophic style approach for nutrient acquisition do not require alive host cells to establish infection, like the biotrophic pathogens do. Instead, it is the other way around; necrotrophic pathogens seem to first kill host cells before they invade. It is believed that any kind of cell death will facilitate necrotrophic colonization, including the cell death associated with the hypersensitive immune response. Although evidence supporting this has been published, much of it is from Arabidopsis and only against a couple of pathogens, such as Botrytis and Sclerotium sp. Indications do exist that it may not be true for all necrotrophic pathogens to be able to exploit HR cell death for colonization. For instance, none of the maize disease lesion mimic mutants have been seen to display any susceptibility to CCR1. This however is based solely on cursory observations. To address it experimentally, use of an R gene mutant (*Rp1-D21*) that triggers HR on its own in the absence of pathogens was utilized to determine resistance and susceptibility. The *Rp1-D21* mutant gene was introgressed into B73 plants carrying or lacking the functional *Hm1* gene, and the plants were inoculated with CCR1 at different stages of development. Interestingly, the HR lesions associated with the expression of *Rp1-D21* had no impact on maize's interaction with CCR1. Plants containing *Hm1* were always resistant, regardless if *Rp1-D21* was in the background or not. Likewise, *Rp1-D21* also failed to impact the susceptible reaction of plants lacking a functional h*m1*. Both the *Rp1-D21*-lacking and Rp1-containing *hm1hm1* plants were equally susceptible to CCR1. This result is interesting given that the plants containing an *Rp1-D21* allele have their defense response genes already induced.

Introduction

Plants are surrounded by an array of pathogens but it is rare to see plants overcome by disease (Anderson, 2010). This is mainly due to the high level of inducible defense mechanisms present

in plants. One of the main contenders in plant defense is the hypersensitive response (HR) it has long been linked to helping plants defend against invading pathogens including; bacteria, fungi, viruses, nematodes, and even insects (Baldwin, 2010). The hypersensitive response is characterized by a rapid cell death around the site of infection that helps to stop the spread of a pathogen. HR is most specifically associated with race specific biotrophs due to their need for living tissue to survive. HR is generally less effective against necrotrophic pathogens due to their requirement for dead host tissue (Balint-Kurti, 2019). Activation of dominant R genes are usually what mediate the response through indirect or direct detection of specific pathogen effector proteins (Bent and Mackey, 2007). Plants have developed these mechanisms to recognize pathogens to mediate their response because over activation can severely compromise the plants energy resources and in some cases can lead to its demise (Freeman 2008). Mutations in R genes have been discovered that get rid of the dependence on effector proteins and allow HR to be triggered constitutively in the absence of a pathogen (Zhang et al., 2003).

The *Rp1* locus in maize is found on chromosome 10 and carries a number of tandemly-repeated paralogs with coiled-coil nucleotide -binding site leucine-rich repeat (CC-NBS-LRR) domains. These domains in maize are known to confer resistance to specific races of common rust caused by the fungus *Puccini sorghi* (Hulbert, 1997). The NLR protein (*Rp1-D21*) is derived from intragenic recombination between the two NLR's *Rp1-D* and *Rp1-dp2* (Wang 2015). *Rp1-D21* a partially dominant autoactivate R gene causes spontaneous formation of HR lesions on stalks and leaves in the absence of pathogen recognition. This spontaneous HR formation is caused by the standard recognition and elicitation function being uncoupled (Olukolu, 2014). HR conferred by *Rp1-D21* is dependent upon light and temperature which is typical of R-genes associated with HR (Balint-Kurti, 2019). The HR conferred includes the accumulation of salicylic acid (SA), reactive oxygen species, and expression of pathogenesis-related genes that is typical of pathogen mediated HR (Olukolu, 2014). Four genes are activated downstream of the *Rp1-D21* defense response, namely Pr1, Pr5, PRms, and Wip1 (Chintamanani, 2010). Induction of these genes was seen in plants displaying the *Rp1-D21* phenotype and were absent in the wt plants, providing evidence that the lesions present are HR lesions (Chintamanani, 2010). SA induces defense against biotrophs while jasmonic acid (JA) activates defense against necrotrophs (Speol, 2007). SA and JA are inversely related in plant defense response, if one is induced it is antagonistic towards the

other (Mur, 2006). In Arabidopsis the growth of the necrotroph B. *cinerea* was suppressed in dnd1 an HR deficient mutant. When dnd1 was exposed to avirulent P *syringae* that induces the SA mediated defense response in turn reduces the JA defense response the invasion of B. *cinerea* was enhanced by the HR conferred by P. syringae (Govrin, 2000). This displays two things; the absence of the dead tissue from HR deterred the growth of B. cinerea, and inducing HR to create dead tissue benefited its colonization. This displays that HR can aid in the ability of necrotrophs to colonize the plant. Expression of the aspartic protease gene AP13 in grapes improves resistance to powdery mildew but decreases resistance to B. *cinerea* in Arabidopsis (Guo, 2016). This finding suggested that *Ap13* suppresses the JA signal transduction pathway while promoting the SA dependent signal transduction pathway and this increases the ability of the necrotrophic pathogen B. *cinerea* to colonize Arabidopsis. In theory with CCR1 being a necrotroph, its interference with histone deacetylases may interfere with the proper induction of ET/JA-mediated defense responses in the host plant. Young (2008) showed that the application of exogenous HC-toxin after infection of Tox^- strain of CCR1, shuts down expression of defense genes.

Necrotrophs rely on the presence of dead or dying tissue that they necrotize themselves or opportunistically feed on saprophytically to grow and reproduce. Many necrotrophs induce necrosis by secreting phytotoxins that work to degrade cell walls (Laluk, 2010). Cell wall degrading enzymes work to open the cell and leak it contents to allow the pathogen to feed off of it. Bacterial necrotrophs like *Pectobacterium carotovorum* are known to secrete various cellulases and proteases to degrade the cell wall (Barras et al., 1994). Such bacteria also produce harpins act as protein elicitors of the hypersensitive response. *P. carotovorum* transports harpins through the type II/III secretion system to directly trigger HR in Arabidopsis (Sandkvist, 2001). Applying harpin exogenously to plant leaves is shown to induce typical HR (Wei, 1992). The production and use of harpins to incite HR are evidence that necrotrophs may be using the dead tissue associated with HR to their advantage (Laluk, 2010). In the case of *B. cinerea* infection in Arabadopsis, wounding prior to inoculation promotes a strong defense against *B. cinerea* known as wound-induced immunity (Mingiste, 2012). The leaked nutrients and open sites for infection is contrary to other pathosystems that use wounding to increase colonization. CCR1 generally utilizes wounds to colonize the plant and reproduce but from the information about *B. cinerea* and

wound-induced immunity we can hypothesize that the dead tissue from HR in *Rp1-D21* may change the resistance response in of maize to CCR1.

Rp1-D21 displays the typical hallmarks of pathogen induced HR which allows us to study the interaction between HR and necrotrophic pathogens. If the programmed cell death tissue associated with HR and the reduction in SA is beneficial to necrotrophs we hypothesize that this programmed cell death and reduction in SA by *Rp1-D21* may change the resistance response to CCR1. To test this, we inoculated B73 plants carrying the autoactive NLR gene *Rp1-D21* and rated the plants on a 1-10 scale to maturity, 1 being completely resistant and 10 being completely susceptible. We used dominant *Hm1* as our resistant control scoring a 1 throughout, and *hm1* as our susceptible control scoring a 10 throughout the experiment. Our data reveals that the HR triggered by the autoactive NLR gene *Rp1-D21* carrying dominant *Hm1* does not change the resistance of *Rp1-D21* to CCR1. Resistance given by dominant *Hm1* is robust enough stop CCR1 from invading. We provide evidence that *Rp1-D21* does not add resistance to lines carrying the CCR1 susceptible allele hm1. From the supporting evidence this conclusion comes as a surprise, but not an unlikely outcome because one copy of dominant *Hm1* is enough for all grasses to defend against CCR1. Dominant *Hm1* unlike many other R genes has been able to withstand the test of time and is not susceptible to boom and bust cycles like many other R genes that have been implemented in maize (Sindhu, 2008).

Results

The presence of Rp1-D21 does not change resistance or susceptibility to CCR1

Studies have measured how lesion mimic mutants affect a plants ability to defend against necrotrophic pathogens like *Botrytis cinerea* (Govrin, 2000), the knowledge gap on their importance to plant pathology warrants further study. We rated plants resistant and susceptible to CCR1 carrying *Rp1-D21* to maturity and compared them to resistant and susceptible varieties in the absence of *Rp1-D21*. Plants inoculated with 300 μL of 50,000 spores/mL 3 weeks after planting were rated every week until maturity at week 7. Over the course of the growing season we did not document any notable decrease in resistance in plants carrying *Rp1-D21* and dominant *Hm1*. We also did not document any increase in susceptibility in plants with the susceptible

background of *hm1*. Plants lacking dominant *hm1* carrying *Rp1-D21* resembled their susceptible counterparts that lack dominant *hm1* and *Rp1-D21*. Our results displayed no decrease in resistance which contradicts the change in resistance seen in Arabidopsis by Chassot et al. when inoculated with *S. sclerotiorum* that produces HR and decreases its resistance to *B. cinerea*. Plants carrying *Rp1-D21* developed necrotic lesions characteristic of this lesion mimic mutant around 3 weeks and continued to develop until maturity. No lesions resembling CCR1 were seen in plants carrying dominant *Hm1* or recessive hm throughout their life cycle, outside of the initial resistance response to inoculation. This determines that the autoimmune HR response and the accumulation of SA that leads to a reduction in JA in maize conferred by *Rp1-D21* does not aid in the colonization of the necrotrophic pathogen CRR1. Previous studies demonstrate that susceptibility to CCR1 is directly correlated with the inability of maize to reduce HC-toxin (Johal and Briggs, 1992).

Plants lacking a dominant copy of *Hm1* in this study were immediately susceptible to CCR1. Plants carrying Hm1 in the presence of *Rp1-D21* displayed minor chlorotic flecking that is typical of a resistance response to inoculation. Figure 4.1 and 4.2 display the resistance phenotype at week 3 and at week 7 where there is no sign of pathogen colonization. Figure 4.3 shows the comparison of lesion ratings on the four chosen phenotypes and displays that there is no change in the resistance or susceptibility of plants carrying *Rp1-D21* and plants lacking *Rp1-D21* throughout the duration of this study.

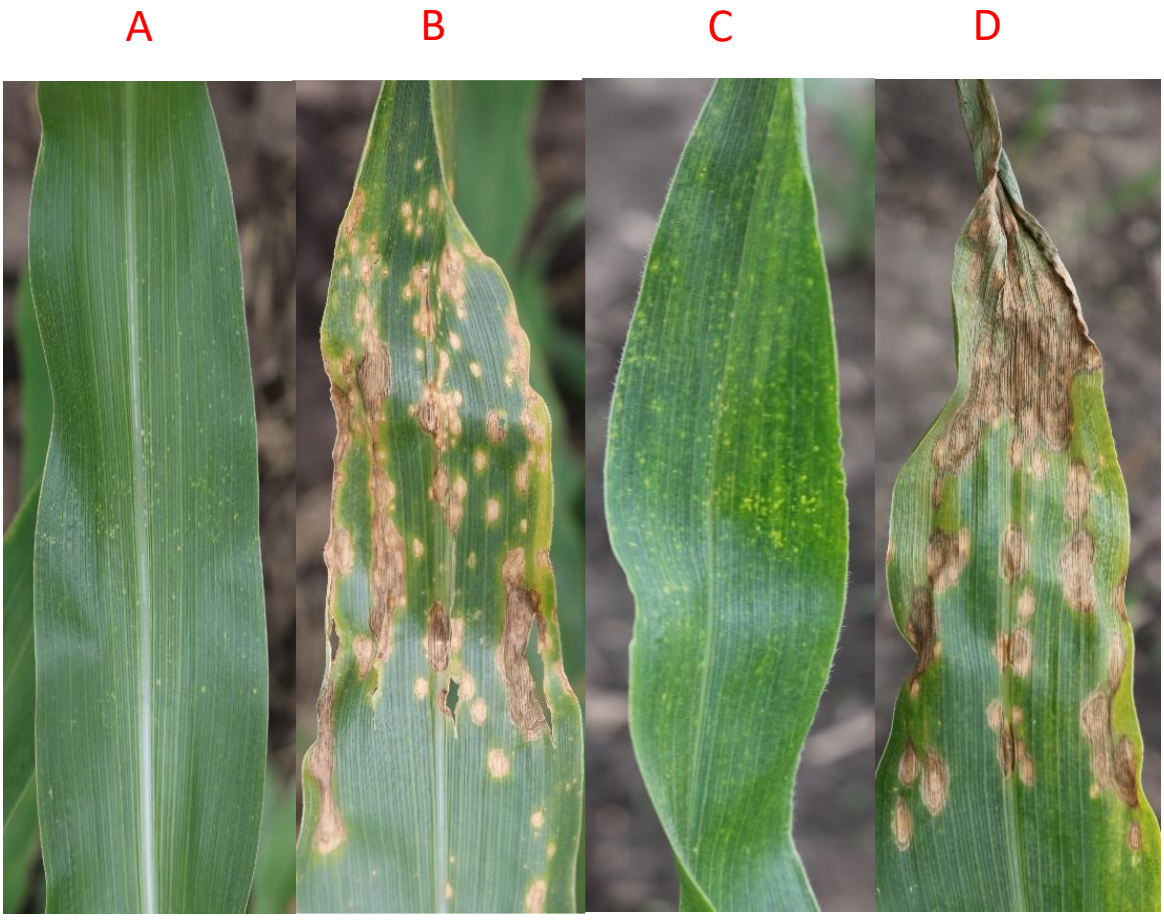

Figure 7. Response of resistant and susceptible cultivars at 3 weeks after planting.
(A) *Hm1-1* resistant control (B) *hm1-2* susceptible control (C) *Rp1-D21* carrying dominant Hm1
(D) *Rp1-D21* carrying recessive *hm1*

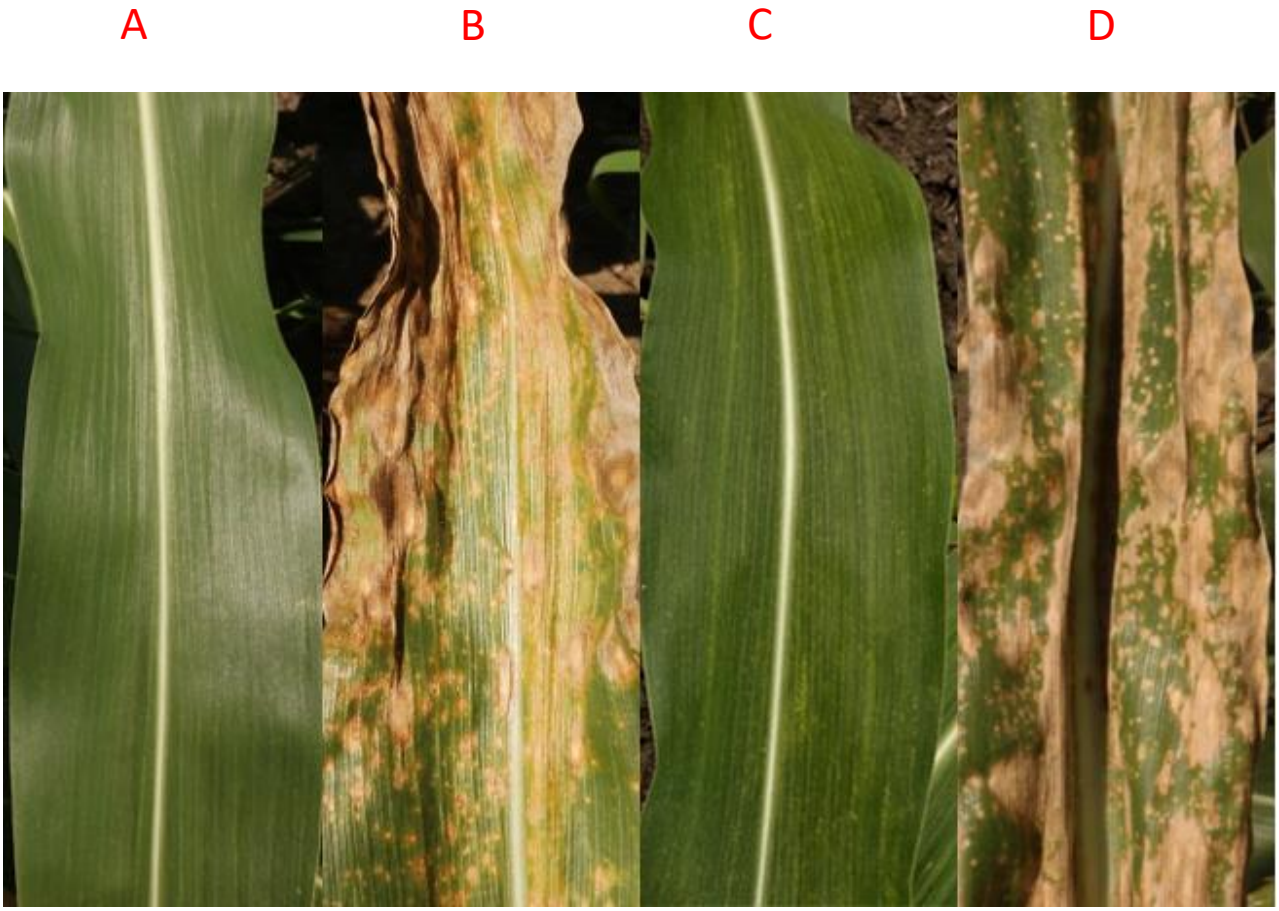

Figure 8. Response of resistant and susceptible cultivars at 7 weeks after planting.
(A) *Hm1-1* resistant control (B) *hm1-2* susceptible control (C) *Rp1-D21* carrying dominant *Hm1*
(D) *Rp1-D21* carrying recessive *hm1*

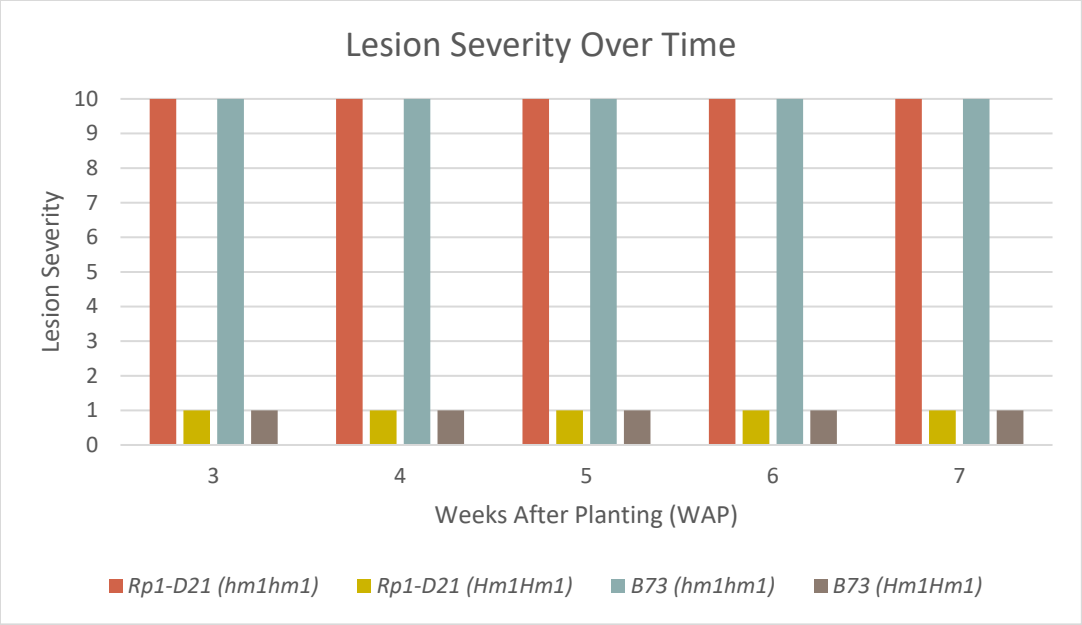

Figure 9. Comparison of the relative strength of plants carrying *Rp1-D21* over time. Hm1-2 and *hm1-2* were the resistant and susceptible controls.

Discussion

There have been few studies testing the resistance and susceptibility of lesion mimic mutants since their discovery in recent years, our study is unique in that we test the disease response of a lesion mimic mutant that displays autoactive HR by inoculating it with the necrotrophic pathogen CCR1. Other studies have shown that *B. cinerea* can utilize the dead tissue from an HR response produced by *P. syringae* (Govrin, 2000). *Cochliobolus victoriae* a necrotroph that produces victorin causing leaf blight in oats and can kill the whole plant. Victorin binds to TRX-h5 activating the LOV1 gene which produces the hypersensitive response and is responsible for the susceptibility to C. *victoriae* (Sweat, 2007). This directly tells us that C. *victoriae* is utilizing the dead tissue associated with the hypersensitive response to its advantage to help it colonize Arabidopsis. It comes as a surprise that we did not see a similar response when inoculating *Rp1-D21* with CCR1. Why CCR1 was not able to utilize the available dead tissue from *Rp1-D21's* autoactive HR response to colonize it remains unclear. This unique glimpse into *Rp1-D21* with a background in B73 carrying dominant *Hm1* not only helps us understand the activity of autoactive HR on necrotrophic resistance but it helps us learn more about the resistance conferred by *Hm1*. The increased SA response in *Rp1-D21* favoring biotrophic resistance reduces the JA response that helps mediate defense responses mainly against necrotrophs, has no effect on maize's ability to

defend against CCR1. JA plays an important role in response to tissue wounding and acting to slow down growth and redirecting metabolism to the production of defense molecules (Larrieu, 2016). A reduction in the ability to mount defenses against tissue wounding should ultimately give CCR1 the ability to colonize *Rp1-D21*. The HC-toxin reductase deployed by *Hm1* is still able to reduce enough toxin to confer resistance. It is apparent that both the resistant and susceptible varieties carrying *Rp1-D21* reacted identically to their counterparts without *Rp1-D21*. This displays that even without dominant *Hm1* autoactive HR does not provide an avenue to increased resistance. Our results display that the *Rp1-D21* autoactive HR has no effect on the maize *Cochliobolus carbonum* race 1 pathosystem. It does not tell us about the molecular mechanism that determine this interaction. A deeper look at other HR lesion mimics in maize may tell us more about their interaction and possible effects while staying inside the well known pathosystem that is the maize *Chochliobolus carbonum* race 1 interaction.

The HR lesion mimic *Rp1-D21* has given us insight on how HR interacts with the necrotrophic pathogen CCR1. This significant finding contradicts the findings of Govrin et al. and Sweat et al. because HR did not significantly decrease resistance to CCR1 carrying dominant *Hm1*. Wound-induced immunity also did not provide increased resistance to CCR1 colonization in susceptible maize carrying *Rp1-D21*. This is contrary to the findings of Chassot et al. because they observed an increase in resistance when plants were wounded before *B. cinerea* inoculation. Wounding opens cells and potentially kills them which benefits necrotrophs and is not known to induce any necrotrophic specific defense responses (Stone, 2001) The reason for this variation in response could be the differences between these pathosystems. Botrytis is a broad host range pathogen where CCR1 has a narrow host range only infecting members of the poaceae family. The difference in host range may imply the involvement of different resistance mechanisms used to fight each necrotroph that vary along with differences in the pathogens themselves. HR triggered by the biotroph involved in the Govrin et al. study certainly has a varied response to the autoactive HR seen in *Rp1-D21*. Our results differing from Govrin et al. results reinstates the complexity of the HR and the variance between pathosystems.

Future Directions

Our findings offer additional evidence to support the hypothesis that developmental changes in the plant via the grain fill period does reverse the resistance gained from the juvenile state to the mature state. This suggests that the metabolic state of the plant and the NADPH pool may have an influence on the ability of different HCTR's of APR alleles to reduce HC-toxin in an efficient manner. The reduction in resistance seen in APR alleles after fertilization would definitely make it worthwhile to quantify NADP(H) levels after fertilization to observe a possible correlation between these two factors. Determining the levels could help warrant further study in the availability of NADP(H). Due to the highly compartmentalized nature of NADP(H) it would be important to determine how much is available for HCTR use. Determining the availability of NADP(H) for HCTR before and after fertilization will be the ultimate deciding factor into understanding the role of NADP(H) in APR alleles.

In the field we also observed that the change in resistance after the grain fill period was initiated also gave the ability for other pathogens to colonize APR alleles. One particular pathogen present was grey leaf spot, it seemed to follow the disease path of CCR1. It began at the bottom of the plant and began to work its way up the plant. This eludes to the possibility that increased susceptibility to CCR1 may also lead to increased susceptibility to grey leaf spot. It would be worthwhile to determine the presence of grey leaf spot as well as CCR1 during and after the grain fill period. The addition of a study rating susceptibility to grey leaf spot on APR alleles of maize could help determine any increase in susceptibility in APR alleles as well as any avenue that CCR1 may open to help other pathogens colonize maize.

Lesion mimic mutants have provided us with a new and exciting way to observe the effects of HR on necrotrophic pathogens. Our results provide additional evidence on the robustness of *Hm1* in maize along with insight on the effects of HR on CCR1 colonization. We determined that maize carrying *Rp1-D21* does not affect resistance to CCR1. Looking further into other mutants like Les23 that carry a similar autoactive NLR caused by Slm1 producing HR that matches the HR of *Rp1-D21* could help determine the effects in a different background (Zhan, 2017). This makes Les23 a good candidate to study the interaction between autoactive HR and *C. carbonum.* The knowledge gap between the effects of HR on necrotrophs is still unclear. Using other mutants in

a well characterized pathosystem like the maize-*cochliobolus carbonum* interaction could simplify determining the molecular basis.

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