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Interactions Between Resistance Genes In Wheat *Triticum aestivum* L. And Wheat Curl Mite Populations *Aceria tosichella* Keifer (Eriophyidae)

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INTERACTIONS BETWEEN RESISTANCE GENES IN WHEAT *TRITICUM AESTIVUM* L. AND WHEAT CURL MITE POPULATIONS *ACERIA TOSICHELLA* KEIFER (ERIOPHYIDAE)

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Wheat curl mite (WCM) (*Aceria tosichella* Keifer) is a major pest of winter wheat (*Triticum aestivum* L.), being the only known vector of three damaging plant viruses, *Wheat streak mosaic virus, Triticum mosaic virus,* and *High Plains wheat mosaic virus*. This wheat-mite-virus complex causes significant yield loss globally. Management has been mostly through cultural practices to reduce mite build up in volunteer wheat, thereby reducing the spread of viruses. Host plant resistance to WCM has also been used as an important management strategy for this wheat-mite-virus complex. However, WCM is a cryptic species complex, resulting in great variability in WCM responses to resistance genes in wheat. Also, the stability of WCM resistance has been questioned because of previous adaptation to one mite resistance gene (*Cmc3*).

Changes in virulence of mite populations were examined after field selection and long-term (i.e., 6-8 months and 12 months) exposure to different mite-resistant wheat varieties TAM 107 (*Cmc3*), TAM 112 (*Cmc3+Cmc4*) and Byrd (*Cmc4*). Mite populations were allowed to go through multiple generations on resistant varieties to estimate their adaptation potential. Mite population counts and leaf curling symptoms were evaluated

after short (14 days) and extended (28 days) mite infestation to estimate the stability of antibiosis and tolerance traits. Results indicate that the effectiveness of antibiosis on WCM populations was reduced with long-term mite exposure to TAM 112 but not for Byrd. This adaptation to the resistance in TAM 112 was only evident for the 12-month colony at the extended 28-day test period. In contrast, plant tolerance remained stable and effective throughout the 12-month colony period.

The transcriptome-level responses of wheat to continued mite feeding and exposure of subsequent mite generations to plant defenses were examined. Results indicate potential mechanisms of resistance for Byrd containing the *Cmc4* gene. Action of phytohormones, combined with lipid signaling and membrane integrity appear to play a role in response to WCM after 10-day-post-infestation (dpi). A higher number of molecular functions are activated at 10 dpi compared to previous work done at 1 dpi for this resistant variety. In addition, the importance of the genes located in the sub-genome D of the wheat in response to mite feeding is identified.

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CHAPTER 1

LITERATURE REVIEW

Introduction

The wheat curl mite (*Aceria tosichella* Keifer, WCM) is an economically significant pest of winter wheat (*Triticum aestivum* L.) in many regions of the world. WCM reduces wheat yield through direct feeding, but its primary impact is through the transmission of several viruses to wheat. WCM feeding damages leaf epidermal tissue, impacting the leaf's ability to unfurl. Mite-infested leaves tend to have their edges curled tightly inward. Non-viruliferous WCM can cause yield loss up to 15% in infested wheat fields (Harvey et al., 2000). In North America, WCM is the vector of three viruses, *Wheat streak mosaic virus* (WSMV), *High Plains wheat mosaic virus*, and *Triticum mosaic virus* (Slykhuis 1955; Seifers et al., 1997; Seifers et al., 2009). Virus co-infections are commonly found in fields across the Great Plains (Burrows et al., 2016). The wheat-mite-virus complex is the third largest cause of yield loss in winter wheat production in Kansas over a 20-year period (Appel et al., 2015). Virus-infected fields commonly result in yield losses up to 100%.

WCM Taxonomic History

The wheat curl mite belongs to the family Eriophyidae and is distributed worldwide (Oldfield & Proeseler, 1996). Due to their small size and the existence of morphologically similar species, accurate identification of eriophyoid mites is difficult. Because of previous taxonomic confusion, WCM can be found in the literature with several taxonomic synonyms including *Aceria tulipae*, *Eriophyes tulipae, Aceria tosichella,* and *Aceria tritici*. WCM was initially misidentified as the dry bulb mite *A.*

tulipae (Keifer, 1938; Slykhuis, 1955). Since host-plant specificity is a common character of many eriophyoid mites, *A. tulipae* was considered to have an unusually wide host range leading to further investigation. Shevchenko et al. (1970) described the mites on wheat as a new species, *A. tritici*. Prior to this, in 1969, mites with identical morphology were found in Serbia and described by Keifer (1969) as *A. tosichella*. Thus, *A. tosichella* takes priority. Unfortunately, the issue was further confounded when *Aceria* was reassigned to the genus *Eriophyes* in 1971 (Newkirk & Keifer, 1971). In 1989, the International Commission of Zoological Nomenclature restored the former uses of the disputed genera, and WCM became correctly referred to as *Aceria tosichella* in the literature (Amrine & Stasny, 1994).

Molecular approaches using DNA-based techniques revealed that WCM is a cryptic species complex (Carew et al., 2009; Hein et al., 2012; Skoracka et al., 2012). Different lineages are distinguishable using DNA sequences from mitochondria (mtDNA COI, 16S) and nucleus (28S rDNA D2, ITS1–ITS2, and ANT). In Poland, multiple genotypes have been found, but only two of those exist in North America. These two most polyphagous and widespread WCM genotypes associated with wheat are known as Type 1 and Type 2 in North America and Australia (Carew et al., 2009; Hein et al., 2012), and as MT-8 and MT-1, respectively, in Europe (Skoracka et al., 2014). WCM Type 1 and Type 2 have been found coexisting in mixed populations, even in a single wheat field, in North America, Australia, and Europe (Siriwetwiwat, 2006; Schiffer et al., 2009; Hein et al., 2012; Skoracka et al., 2017).

WCM Biology and Ecology

WCM success as a key pest is favored by its many biological traits that support it as an r-selected species (Speight et al., 2008). These include small body size, secluded habitats, short generation time, high offspring production, and high dispersal ability. WCM are white in color, cigar shaped with four legs near the front end, and measure 170-250 microns in length (Keifer, 1939). The mouthparts consist of a two-lobed rostrum with a pair of stylets that are slightly curved, needle-like structures (Orlob, 1966). The stylets are about 20 microns in length, but only about one-third of the total length (~5 microns) is involved in penetrating the plant tissues (Orlob, 1966; Royalty & Perring, 1996). Thus, WCM can only feed on the epidermal cells.

After infesting wheat plants, WCM move down the plant to the base of the leaf sheath (Somsen & Sill, 1970). WCM feed between the leaf veins in grooved sections occupied by bulliform cells (Orlob, 1966). Bulliform cells are known to be mainly water-containing and to be poor in solid contents. The protoplasmic layer of the cell wall may be another food source for WCM (Orlob, 1966). Damage to bulliform cells impacts the ability of the leaves to unfurl, resulting in curling or rolling at the edge of the leaf, and this can lead to entrapment of the subsequent emerging leaf (Orlob, 1966; Styer & Nault, 1996). The leaf curling creates a favorable microenvironment for the mites to survive, colonize, and shelter from environmental impacts and miticidal exposure. Beside distortion of plant or leaf growth, plant damage from feeding includes the withdrawal of nutrients, reduction of gas exchange and photosynthesis, and even death of epidermal cells (Sabelis & Bruin, 1996).

The WCM completes its life cycle in 7-10 days at 24-25°C with four stages: egg, first nymph, second nymph, and adult (Slykhuis, 1955; Staples & Allington, 1956). At 25°C and a relative humidity of 100%, the majority of eggs hatch in approximately four days and first nymphs emerge (Slykhuis, 1955). Each immature stage takes approximately 36 hours at 25°C. Prior to each molt, there is a quiescent phase for about 18 hours where the mites are incapable of movement and appear translucent anteriorly and sometimes posteriorly (Staples & Allington, 1956). After an adult emerges, it requires an additional 1 to 2 day preoviposition period. Without a host, WCM survival highly depends on temperature and humidity. As temperature increases, mite survival decreases (106 h at 10°C vs 17 h at 30°C)(Wosula et al., 2015). At 25°C and 95% relative humidity, mites can survive 40 h off a living host. However, with relative humidity at 2%, mites can only survive 9.5 h at 25°C.

WCM reproduce via indirect sperm transfer (Oldfield, 1970). Spermatophores containing sperm are placed by the males on the plant surface, and these are picked up by the females (Lindquist et al., 1996). WCM reproduce through arrhenotokous parthenogenesis, in which unmated females produce haploid eggs that develop into males, while mated females produce diploid eggs that develop into females (Helle & Wysoki, 1983). A WCM female can produce at least 12-25 eggs in her lifetime (Staples & Allington, 1956; Salome et al. 1964). Under optimal conditions, a single female mite is estimated to have over 3 million descendants in 60 days (Somsen & Sill, 1970). The temperature ranges for population growth are 10.4 to 35.7°C for Type 1, and 12.2 to 40°C

for Type 2 (Kuczyński et al., 2016). Optimal temperatures are about 32°C and 35°C for Type 1 and 2, respectively.

Later in the growing season, mites move to florets and feed on developing kernels. When the flag leaves and the heads are drying, WCM start moving to the outer surfaces of the wheat head for dispersal (Nault & Styer, 1969). Although plant deterioration is a factor for dispersal, the main influence on the level of dispersal is related to the size of the source population (Thomas & Hein, 2003). WCM have several modes of dispersal including walking, phoresy, and aerial dispersal (Slykhuis, 1955; Gibson & Painter, 1957; del Rosario & Sill, 1964). Of these three, wind plays an important role in WCM dispersal. To initiate wind dispersal, WCM move to exposed areas on the plant, crawl upon one another, attach through their caudal suckers to form chains, and wait for the wind to carry them away. However, these pre-dispersal behaviors are not a prerequisite for wind dispersal (Laska et al., 2019). WCM can disperse at any wind speed, but higher wind speeds (>9m/s) can increase the dispersal distance (Stilwell et al., 2019). WCM can disperse in significant numbers up to 3.3 km from the source field.

Mite-Virus Management

Current management tactics are based on an Integrated Pest Management (IPM) approach that combines cultural practices (i.e., manage volunteer wheat and use of optimal planting dates) and host plant resistance to both mites and viruses. No effective acaricides have been identified for this complex (Murphy & Burrows, 2021). While

sources of virus resistance in wheat have recently been identified, incorporation into commercial wheat faces challenges with resistance breaking down as temperature increases (Nachappa et al., 2021). Thus, reducing mite vector population is crucial for managing the disease complex.

Pre-harvest volunteer wheat serves as a primary ‘green bridge’ refuge for WCM between summer harvest and fall planting (Wegulo et al., 2008). However, effective control of the ‘green bridge’ is not always feasible because WCM can utilize over 90 other grass species and disperse by wind up to 3.3 km from source field (Navia et al., 2013; Stilwell et al., 2019). Delayed planting can also reduce fall infections by shortened exposure periods between the wheat crop and alternate hosts (Hunger et al., 1992; McMechan & Hein, 2016; Wosula et al., 2018). However, planting dates are typically determined by soil moisture in dryland production systems and large farm size can also create time constraints to delay planting. Taken together, use of mite-resistant wheat varieties could serve as an effective strategy to reduce WCM occurrence, and the spread of viruses. However, the stability and sustainability of resistance genes is a concern with the history of mite populations overcoming resistance (Harvey et al., 1997). Furthermore, the mechanisms underlying WCM–wheat interactions are not well known. There is a need for a greater understanding of wheat defense responses and adaptation ability of WCM to these defenses.

Host Plant Resistance against WCM

Plants are thought to have three major resistance categories: antixenosis, antibiosis, and tolerance (Painter, 1951; Smith, 2005). Antixenosis plant traits adversely affect arthropod behavior, leading to reduce colonization or acceptance of a plant as a host. Antibiosis describes adverse effects of resistant plants on herbivore physiology and life histories such as reduced growth, survival, and fecundity. Tolerance is the ability of plants to withstand or compensate for arthropod injury to a degree exceeding susceptible plants.

To date, four different wheat curl mite colonization (*Cmc*) genes have been identified and transferred from wild relatives of wheat. *Cmc2* was transferred from *Agropyron elongatum* (Host) Beauv. to the wheat chromosome 6DL (Andrews & Slykhuis, 1956; Martin et al., 1976; Whelan & Hart, 1988). *Cmc3* was transferred from rye (*Secale cereale* L.) to the wheat chromosome 1AL and released commercially as 'TAM 107' (Martin et al., 1984; Whelan & Hart, 1988; Malik et al., 2003a). However, the extensive planting of 'TAM 107' during the 1980's into the mid 1990's led to WCM adaptation and loss of effectiveness of the gene (Harvey et al., 1997). *Cmc1* and *Cmc4* were both transferred from *Aegilops tauschii* (Coss.) Schmal (Thomas & Conner, 1986; Whelan & Thomas, 1989; Malik et al., 2003a). Despite being on the chromosome 6DS in wheat, *Cmc1* and *Cmc4* were previously designated as independent (Malik et al., 2003b). WCM resistance in wheat variety 'TAM 112' was also mapped in the chromosome 6DS and reported as Cmc_{TAM112} (Dhakal et al., 2018). However, Cmc_{TAM112} was found to be located closely or overlapped with *Cmc4*, suggesting that they are likely to be the same

gene (Zhao et al., 2019). Indeed, a recent study showed that *Cmc1*, *Cmc4*, and Cmc_{TAM112} all shared the same resistance haplotype, indicating that they are the same gene (Silva, 2021).

Genes conferring resistance against WCM are mainly characterized by the average number of mites and leaf symptoms rating after short-term (7-14 days) exposure on the plants (Harvey et al., 1995b, 1999, 2001; Malik et al., 2003b; Dhakal et al., 2017; Carver et al., 2016; Khalaf et al., 2019; Zhao et al., 2021). Compared to susceptible plants, WCM population size and leaf curling symptoms were reduced in resistant plants (Thomas & Conner, 1986; Murugan et al., 2011). By inhibiting the reproductive capacity of WCM, resistant wheat also helped reduce the spread of WSMV (Conner et al., 1991; Harvey et al., 2005). Mite-resistant varieties reduced the incidence of WSMV in the field by 58% and the transmission of WSMV in the greenhouse by 74% (Martin et al., 1984). Seven accessions of *A. tauschii* were evaluated for different categories of resistance against WCM (Carrera et al., 2012). No-choice assay showed antibiosis in four accessions with low mite population after 20 day-post-infestation (dpi). Tolerance to WCM was found in three accessions and the mite-susceptible variety using tolerance index values and dry biomass loss comparisons. Choice assays showed antixenosis in four accessions with reduce number of mites and leaf curling.

While a categorical scale of leaf symptoms and number of mites present can indicate some type of resistance, the actual plant response to the mites is still largely unknown. The mechanisms by which *Cmc* genes contribute to plant defense are unknown. Plant defense responses to arthropods are a combination of constitutive

defenses (i.e., preexisting, always present on a plant) and induced defenses (i.e., specifically activated upon an herbivore or pathogen attack). Induced defenses against herbivores can be roughly divided in three steps: (1) recognition of herbivory attack, (2) induction of several defense signals, and (3) defense responses. Kiani et al. (2021) have provided the only study so far to identify potential genes and pathways involved in defense response against WCM herbivory. After 24-hour post infestation, 'TAM 112' wheat plants showed modifications in their transcriptomes through the expression of genes involved in jasmonic acid (JA) defense pathways, WKRY transcription factors, antioxidation processes, and pathogen-related responses. However, these genes were unaffected in the WCM-susceptible variety 'Karl 92'. The long-term effectiveness of host resistance to WCM is challenged by WCM adaptation. In the case of the first commercial WCM resistant wheat variety 'TAM 107' (*Cmc3),* Harvey et al. (1995a) reported that *A. tosichella* developed a resistance-breaking population after being reared on TAM 107 for 2 months in laboratory. Adaptation to TAM 107 in the field was reported with WCM collected in Kansas (Harvey et al., 1995b, 1997).

Harvey et al. (1999) tested WCM populations collected across the Great Plains from 'Nebraska' (NE), 'Kansas' (KS), 'South Dakota' (SD), 'Texas' (TX), and 'Montana' (MT). These mites were placed on wheat varieties with different genes for WCM resistance (*Cmc1, Cmc2,* and *Cmc3).* Mean number of WCM after 8 dpi showed mites from different location varied in their responses to different resistance wheat varieties. WCM virulence responses to wheat resistance genes has repeatedly been shown to be dependent on the source of mites tested (Harvey et al., 1995b, 1999, 2001; Malik et

al., 2003a). Mite populations also differ in their ability to vector viruses (Seifers et al., 2002). Two WCM genotypes were identified from these populations using PCR-RFLP of the mitochondrial cytochrome oxidase subunit I (COI) and cytochrome oxidase subunit II (COII) region and ribosomal DNA (Type 1: SD, KS, TX, MT and Type 2: NE) (Hein et al. 2012). However, both genotypes can be found overlapping their geographic distribution (Siriwetwiwat, 2006).

The currently used resistant wheat variety TAM 112 (*Cmc1/ Cmc4/ Cmc$_{TAM112}$*) and wheat lines with TAM 112 in their pedigrees were tested against Texas WCM collections (Dhakal et al., 2017). Out of 41 lines, only 12 were found resistant to Type 2 mites. Type 1 mites were used for screening but not genetically confirmed due to colony contamination. Another wheat variety OK05312 (*Cmc4)* was characterized for its resistance against WCM based on leaf symptoms and numbers of mites present at 14dpi (Carver et al., 2016; Zhao et al., 2021). Twenty-five WCM populations collected from Kansas, Missouri, Nebraska, Texas, North Dakota, and South Dakota were evaluated against wheat plants containing *Cmc2, Cmc3,* and *Cmc4* (Khalaf et al., 2019). Mite population counts after 14 dpi showed that *Cmc2, Cmc3,* and *Cmc4* plants were resistant to 24%, 56%, and 80% of mite populations, respectively. Some mite populations were significantly higher on *Cmc3* plants than on susceptible control plants.

Host adaptation of WCM creates serious concerns about the stability of resistant varieties and the sustainability of breeding focused on mite resistance. Rapid adaptation of WCM is favored by high reproductive rates and short generation times. WCM showed little impact on reproduction when returning to wheat after rearing on alternative hosts

(barnyard grass, green foxtail, and foxtail millet) for 42 days, except for Type 1 mites on barnyard grass (McMechan, 2016). Recently, WCM Type 2 were shown to adapt to a new host (barley) at the time point of 45 generations (Skoracka et al., 2022). Research is needed to address the long-term reproduction and adaptation of WCM on wheat plants with *Cmc* genes to gain a more accurate evaluation of the stability and sustainability of these resistant varieties. Developing effective and sustainable mite-resistant wheat is a major challenge. A more thorough knowledge of WCM-wheat interactions will allow us to improve the success of host plant resistance strategies and reduce factors responsible for WCM adaptation. In particular, the objectives of this study are (1) to explore the transcriptome-level responses of wheat varieties with mite-resistant genes to continued mite feeding and the exposure of subsequent mite generations to plant defenses resulting from extended mite infestation and (2) to determine the genetic variability and structure of mite populations on resistant wheat varieties in the field and the changes in the virulence of these populations after long-term exposure to mite-resistant wheat varieties.

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CHAPTER 2

STABILITY OF WHEAT RESISTANCE GENES AGAINST WHEAT CURL MITE POPULATIONS AFTER LONG TERM EXPOSURE

Introduction

The wheat curl mite (*Aceria tosichella* Keifer, WCM) is an economically significant pest of winter wheat (*Triticum aestivum* L.) in many regions of the world. WCM reduces wheat yield through direct feeding, but its primary impact is through the transmission of several viruses to wheat. In North America, WCM is the vector of three viruses, *Wheat streak mosaic virus* (WSMV), *High Plains wheat mosaic virus*, and *Triticum mosaic virus* (Slykhuis 1955; Seifers et al., 1997; Seifers et al., 2009). Virus co-infections are commonly found in fields across the Great Plains (Burrows et al., 2016). Virus-infected fields commonly result in yield losses up to 100%.

After infesting wheat plants, the tiny (0.2 mm) WCM move down the plant to the base of the leaf sheath and start feeding (Somsen & Sill, 1970). WCM feed between the leaf veins in grooved sections occupied by bulliform cells. Damage to bulliform cells impacts the ability of the leaves to unfurl, resulting in curling or rolling at the edge of the leaf, and this can lead to entrapment of the subsequent emerging leaf (Orlob, 1966; Styer & Nault, 1996). The leaf curling creates a favorable microenvironment for the mites to survive, colonize, and shelter from miticidal exposure. Beside distortion of plant or leaf growth, plant damage from feeding includes the withdrawal of nutrients, reduction of gas exchange and photosynthesis, and even death of epidermal cells (Sabelis & Bruin, 1996). The pest status of the WCM relies heavily on its high reproductive capacity as it can complete its life cycle in only 7-10 days at 24-25°C (Staples & Allington 1956; Kuczyński et al. 2016). It is estimated non-viruliferous mites can cause yield loss up to 15% in wheat fields (Harvey et al. 2000).

Another challenge in control WCM is that it is a cryptic species complex (Carew et al., 2009; Hein et al., 2012; Skoracka et al., 2012). Two polyphagous and widespread WCM genotypes known as Type 1 and Type 2 have been found associated with wheat in North America and Australia (Carew et al., 2009; Hein et al., 2012), and as MT-8 and MT-1, respectively, in Europe (Skoracka et al., 2014). WCM Type 1 and Type 2 have been found coexisting in mixed populations, even in a single wheat field, in North America, Australia, and Europe (Siriwetwiwat, 2006; Schiffer et al., 2009; Hein et al., 2012; Skoracka et al., 2017).

Current management tactics are based on an Integrated Pest Management (IPM) approach that combines cultural practices (i.e., manage volunteer wheat and use optimal planting dates) and host plant resistance to both mites and viruses. While sources of virus resistance in wheat have recently been identified, incorporation into commercial wheat faces challenges with resistance breaking down as temperature increases (Nachappa et al., 2021). Thus, reducing mite populations is crucial for managing the disease complex. Pre-harvest volunteer wheat serves as a primary ‘green bridge’ refuge for WCM between summer harvest and fall planting (Hein et al. 2022). However, effective control of the ‘green bridge’ is not always feasible because of alternate WCM hosts and the ability of mites to disperse by wind (Navia et al., 2013; Stilwell et al., 2019). Delayed planting can also reduce fall infections by shortened exposure periods between the wheat crop and alternate hosts (Hunger et al., 1992; McMechan & Hein, 2016; Wosula et al., 2018). However, planting dates are typically determined by soil moisture in dryland production systems and large farm size can also create time constraints to delay planting. Taken

together, use of mite-resistant wheat varieties could serve as an effective strategy to reduce WCM occurrence, and the spread of viruses.

To date, four different wheat curl mite colonization (*Cmc1-4*) genes have been identified and transferred from wild relatives of wheat including tall wheat grass (*Agropyron elongatum* (Host) Beauv.; *Cmc2*), rye (*Secale cereale* L.; *Cmc3*), and goatgrass (*Aegilops tauschii* (Coss.) Schmal; *Cmc1* and *Cmc4*) (Andrews & Slykhuis, 1956; Martin et al., 1976; Martin et al., 1984; Thomas & Conner, 1986; Whelan & Hart, 1988; Whelan & Thomas, 1989; Malik et al., 2003a). *Cmc1* and *Cmc4* were previously designated as independent genes (Malik et al., 2003b), and Cmc_{TAM112} was reported as a new WCM resistance gene in wheat variety 'TAM 112' (Dhakal et al., 2018). However, Cmc_{TAM112} was found to be located close to or overlappeding with *Cmc4*, suggesting that they are likely to be the same gene (Zhao et al., 2019). A recent study showed that *Cmc1*, *Cmc4*, and Cmc_{TAM112} all shared the same resistance haplotype, indicating that they are the same gene (Silva, 2021).

Mite-resistance varieties have mainly been evaluated by the reduction of WCM population increases and/or leaf curling symptoms compared to susceptible varieties (Thomas & Conner, 1986; Harvey et al., 1995b, 1999, 2001; Malik et al., 2003b; Murugan et al., 2011; Carver et al., 2016; Dhakal et al., 2017; Khalaf et al., 2019; Zhao et al., 2021). TAM 107 was the first commercially released mite-resistant (*Cmc 3*) variety and widely planted throughout the west-central Great Plains during the late 1980's and 1990's (Porter et al., 1987). By inhibiting the reproductive capacity of WCM, mite-resistant wheat also helped reduce the spread of WSMV (Conner et al., 1991; Harvey et

al., 2005). Mite-resistant varieties reduced the incidence of WSMV in the field by 58% and the transmission of WSMV in the greenhouse by 74% (Martin et al., 1984). However, Harvey et al. (1995a) reported that *A. tosichella* developed a resistance-breaking population after being reared on TAM 107 for 2 months in laboratory. Adaptation to TAM 107 in the field was reported with WCM collected in Kansas (Harvey et al., 1995b, 1997). Harvey et al. (1999) tested WCM populations collected across the Great Plains from 'Nebraska' (NE), 'Kansas' (KS), 'South Dakota' (SD), 'Texas' (TX), and 'Montana' (MT). These mites were placed on wheat varieties with different genes for WCM resistance (*Cmc1*, *Cmc2*, and *Cmc3*). Mean number of WCM after 8 day-post-infestation (dpi) showed mites from these different populations varied in their responses to different resistance genes. Malik et al. (2003b) classified the same NE, KS, and MT populations as 'biotypes' and tested their responses to different accessions of *A. tauschii*. Plants with normal leaves after 7 to 14 dpi were classified as resistant. Hein at al. (2012) characterized the genetic differences of the same five populations by Harvey et al. (1999) into two WCM genotypes (Type 1: SD, KS, TX, MT and Type 2: NE). Notably, the MT population showed a slight but consistent separation from other Type 1 populations. Khalaf et al. (2019) tested twenty-five WCM populations collected from Kansas, Missouri, Nebraska, Texas, North Dakota, and South Dakota against wheat plants containing *Cmc2*, *Cmc3*, and *Cmc4*. Mite population counts after 14 dpi showed that *Cmc2*, *Cmc3*, and *Cmc4* plants were resistant to 24%, 56%, and 80% of mite populations, respectively. Population increases for some mite populations were significantly higher on *Cmc3* plants than on susceptible control plants.

TAM 112, released in 2005, is another popular commercially released mite-resistant variety (Rudd et al., 2014). TAM 112 was one of the top two planted wheat varieties for nearly a decade in Texas and is currently still in the top three (NASS, 2020a). TAM 112 has also been popular in Kansas and Colorado since the late 2000's (NASS, 2016, 2017). Dhakal et al. (2017) tested TAM 112 and wheat lines with TAM 112 in their pedigrees against two Texas WCM collections: TWCMC1 colony (genotype not confirmed due to colony contamination), and TWCMC2 colony (confirmed as Type 2). TAM 112 was found resistant to both colonies. Out of 41 wheat lines with TAM 112 in their pedigrees, 19 were found resistant to TWCMC1 colony. These 19 lines were tested for TWCMC2 colony and only 12 were found resistant. TAM 107 was found resistant to TWCMC1 but susceptible to TWCMC2 colony. Notably, TAM 112 was also found to contain the wheat-rye translocation 1AL.1RS with *Cmc3*. Taken together with other studies, TAM 112 resistance is possibly influenced by both *Cmc3* and *Cmc4* (*Cmc1/* Cmc_{TAM112}). Byrd is a mite-resistant variety resulting from the crossing of TAM 112 and CO970547-7, and was released in 2011 (Haley et al., 2012). Byrd is currently among the top three planted wheat varieties in Colorado (NASS, 2020b). Despite having TAM 112 in its pedigree, Byrd does not possess the wheat-rye translocation 1AL.1RS with *Cmc3* (Dhakal et al., 2017). Byrd was found to be resistant against both Texas mite colonies. Thus, mite-resistance in Byrd is likely derived from *Cmc4* in TAM 112.

More mite-resistant varieties have been developed and released, but adaptation of WCM populations to resistant wheat remains a serious concern with the sustainability of breeding focused on mite resistance. Rapid adaptation of WCM is likely favored by high

reproductive rates and short generation times. WCM showed little impact on reproduction when returning to wheat after rearing on alternative hosts (barnyard grass, green foxtail, and foxtail millet) for 42 days, expect for the reduced reproductive rates of Type 1 mites transferred from barnyard grass (McMechan, 2016). Recently, WCM Type 2 were shown to adapt to a new host (barley) after 45 generations (Skoracka et al., 2022). Research is needed to address the long-term reproduction and adaptation of WCM on wheat plants with *Cmc* genes to gain a more accurate evaluation of the stability and sustainability of these resistant varieties. In particular, the objectives of this study are (1) to determine the changes in the virulence of these populations after long-term exposure to different mite-resistant wheat varieties (2) to determine the genetic variability and structure of mite populations on resistant wheat varieties in the field.

Materials and Methods

Wheat Varieties

Four hard red winter wheat varieties (*T. aestivum* L.) were used in this study. Settler CL is a WCM-susceptible variety. TAM 107 is a WCM-resistant variety carrying the *Cmc3* gene. TAM 112 is a WCM-resistant variety carrying both the *Cmc3* and *Cmc4* gene. Byrd is WCM-resistant wheat variety with TAM 112 as a parent but only carrying the *Cmc4* gene. Four seeds were planted per cone-tainers (3.8 cm top diameter and 20 cm length; Steuwe and Sons Inc., Corvallis, Oregon, USA) containing standard greenhouse mix. Cone-tainers were covered with tube cages and kept in the greenhouse at 24^{o}C (+/- 3^{o}C). Cages were made from clear cylindrical plastic tubes (4 cm diameter and 50 cm length), vented with three 5-cm diameter openings covered with Nitex® screen (80-

micron mesh opening; BioQuip Products, Rancho Dominquez, CA). At 14 days after planting, cone-tainers were thinned to two plants per cone before mite infestation.

WCM Stock Populations

Four WCM stock populations were established in June 2019 and 2020 using WCM collected near Mead, NE. Different field locations were used for each year to increase sampling diversity. In the field, the four wheat varieties were grown in a randomized complete block design with six replications for each variety. For each of the six replicates, five wheat heads from each of the four varieties were randomly selected, cut 1-2 cm below the lowest spikelet, and placed in Ziploc bags on ice. In the laboratory, wheat heads were inspected under a stereomicroscope at 30-40X. Thirty to fifty mites were randomly selected from all five wheat heads from each variety in each rep and transferred to new wheat plants of the same variety to initiate the stock populations.

Mites were transferred using a human eyelash attached to a wooden dowel and placed on a black insect mounting triangle (10 mm x 4 mm). The triangle was then placed in the leaf axil of the new plant. Only adult mites exhibiting active movement were transferred. After infestation, cone-tainers were covered with cages and remained in the lab for a period of 10-15 hours to allow mites to settle on the plants. Cone-tainers were then transferred to a growth chamber with 14:10 (L:D) cycle maintained at 25^{o}C and ca. 60% relative humidity. Mites were transferred to new wheat plants in cones every four weeks to maintain the colony. Mite population, found and constantly reared on Settler CL wheat plants, is referred to as Settler colony. Similarly, mite populations isolated from

and constantly reared on TAM 107, TAM 112, and Byrd wheat plants, are referred to as TAM 107, TAM 112, and Byrd colonies, respectively.

WCM Population Performance on Different Resistant Wheat Varieties

To measure the fitness of mite populations after long term exposure to the same host plant, mite populations were counted at 14- and 28-day post infestation (dpi). For the 2019 populations, experiments were conducted at 6 and 12 months after establishment. For the 2020 populations, experiments were conducted at 8 and 12 months after establishment. Each experiment consists of ten treatments with six replications in a randomized complete block design. Settler colony mites were tested on all four varieties. TAM 107, TAM 112, and Byrd colony mites were tested on their main host and on Settler CL. Ten mites were placed on a paper triangle and the triangle inserted into the whorl of the youngest leaves on each of the two wheat plants in each cone. Mites were transferred and allowed to build up in the similar manner as described above.

At 14 dpi, one plant from each cone was randomly selected, cut at the soil level, and evaluated for leaf curling symptoms on a 0 to 3 scale. For leaf curling rating of each plant, ‘0’ was assigned for no curling, ‘1’ was assigned for slight curling, ‘2’ was assigned for distinct curling, and ‘3’ was assigned for tubular tightly curled leaves with trapping of subsequent leaf. Plants were then placed in a zip-lock bag and refrigerated until mite counting. The remaining plant was collected for counting at 28 dpi. All live mites on each plant were counted under a stereomicroscope at 30-40X. After counting, mites were collected and stored in a petri dish with 100% alcohol for genetic identification later.

WCM Genetic Identification

The protocol for genetic identification is based on methods from Siriwetwiwat (2006) and Hein at al. (2012) with modifications to optimize DNA yield from single mites. Mites collected after counting from all six replications of 6-8-month colonies were used for genetic identification. Single mites were pipetted from a petri dish and placed on the tip of a disposable pestle. Mite was placed and crushed in 0.2 ml PCR reaction tubes containing a mixture of 4 µl of 5X PCR buffer (Promega Cooperation, Madison, Wisconsin USA) and 16 µl of nuclease-free water. Tubes were heated to 99ºC for five minutes for denaturation and then placed on ice. Mixture of 6 µl of 5X PCR buffer, 0.3 µl of *Taq* DNA polymerase, 1 µl of deoxynucleotide triphosphates (dNTPs), 2.5 µl of 10µM primer rDNA2 (TTGATTACGTCCCTGCCCTTT), 2.5 µl of 10µM primer 28Se (CAACTTTCCCTCACGGTACTTG), and 17.7 µl of nuclease-free water were added to each tube to make a final volume of 50 µl. Each tube was vortexed and micro-centrifuged before placing in the PCR thermal cycler (Applied Biosystems^{TM}). Cycling consisted of 12 cycles of 94ºC for 1 min, 52ºC - 41ºC for 1 min (decrease annealing temperature 1ºC per cycle), 72ºC for 2 min, followed by 28 cycles of 94ºC for 1 min, 41ºC for 1 min, 72ºC for 2 min, and 1 cycle of 72ºC for 10 min.

PCR products (8 µl) were analyzed using electrophoresis in a 1% agarose gel. Samples containing visible, single bands were used for restriction fragment length polymorphism (RFLP) analysis. Restriction reactions (20 µl) were carried out using 10 µl PCR products, 2 µl of 10X Buffer C, 0.2 µl of BSA (10 µg/µl), and 0.5 µl restriction enzyme HhaI (10 u/µl; Promega). The mixture was incubated for 2 to 3 hours at 37°C.

The digested PCR products were analyzed by 2% agarose gel electrophoresis and stained with Sybr Safe in 1X TAE for 45 min at 120 volts. Visual analysis of the DNA banding pattern compared to the DNA ladder was used to differentiate WCM Type 1 and Type 2. Digested PCR products of WCM Type 1 showed 5 restriction fragments with estimated size of 520, 340, 320, 300, and 130 base pairs. Digested PCR products of WCM type 2 showed 4 restriction fragments with estimated size of 700, 520, 300, and 130 base pairs.

Statistical Analysis

Mite counts were analyzed using PROC GLIMMIX procedure (SAS® OnDemand for Academics; SAS Institute 2021) to test the fixed effects of year, treatment, colony age, and dpi. Random effects were replication and replication by treatment. Studentized residuals indicated that the data were not normally distributed. Variances increased geometrically as a function of the mean indicating a negative binomial distribution. Mite counts were transformed with the natural log function $\eta=\log(\mu)$ to obtain parameter estimates on the model scale. To interpret results as treatment means, the function $\mu = e^{\eta}$ was used to back transform the model scale to data scale. Bonferroni adjustments were used to account for multiple comparisons and to obtain appropriate p-values.

Leaf curling rating data were analyzed using PROC GLIMMIX and PROC GLM procedures. A multinomial distribution was used to fit the leaf curling rating because this was measured as an ordered ranking. Fixed effects were year, treatment, colony age, and dpi. Random effects were replication and replication by treatment. Least significant mean differences were used to determine differences between main effects. The probability of

each curling rating was reported to determine the frequency of curling symptoms for each treatment and dpi combination.

Results

Mite Population Counts

An analysis of variance (ANOVA) type I test for fixed effects (Table 2.1) indicated that there were significant treatment and year differences in mite presence, but there was no significant treatment by year interaction. Therefore, data from both years were combined for further analyses. In the analysis of variance (Table 2.2), the main effects of treatment ($F_{9,99}$=11.46, P<0.0001), colony age ($F_{1,330}$=181.72, P<0.0001), dpi ($F_{1,330}$=2799.77, P<0.0001), colony age by dpi interaction ($F_{1,330}$=4.98, P=0.0263), and their three-way interaction ($F_{1,330}$=2.26, P=0.0184) were all significant. The significant increase in mite presence for the 28 dpi treatments was expected because of the additional time for mite population buildup. Because of the significant interactions with dpi, data were analyzed separately for 14 dpi and 28 dpi. To further identify the source of the significant interactions, for each resistant variety (TAM 107, TAM 112, Byrd), the mean number of mites found with each treatment combination of two mite colony populations (Settler CL + resistant variety) and two host populations (Settler CL + resistant variety) were analyzed separately.

TAM 107 (Cmc3) vs Settler CL (no Cmc): Four treatments (Settler CL colonies on Settler CL and TAM 107, and TAM 107 colonies on Settler CL and TAM 107) were analyzed separately at 14 and 28 dpi. ANOVA Type III fixed effects (Table 2.3) at 14 dpi indicated that colony age had a significant effect ($F_{1,44}$=35.91, P<0.0001), while treatment

and treatment by colony age interaction had no significant effect. At 14 dpi (Fig 2.1A), 12-month colonies produce significantly more mites than 6-8-month colonies in all treatments, except TAM 107 colonies on TAM 107. At 28 dpi, mite populations from the 12-month colonies remained higher than the 6-8-month colonies ($F_{1,44}$=26.45, P<0.0001) (Table 2.3), and the treatment by colony age interaction was significant ($F_{3,44}$=3.14, P=0.0345), while treatment had no significant effect. At 28 dpi (Fig 2.1B), only 12-month TAM 107 colonies on both Settler CL and TAM 107 produced significantly more mites than 6-8-month colonies.

TAM 112 (Cmc3 + Cmc4) vs Settler CL (no Cmc): Four treatments (Settler CL colonies on Settler CL and TAM 112, and TAM 112 colonies on Settler CL and TAM 112) were analyzed separately at 14 and 28 dpi. ANOVA Type III fixed effects (Table 2.4) indicated that at both 14 and 28 dpi, treatment ($F_{3,33}$=10.80, P<0.0001; $F_{3,33}$=9.15, P=0.0002, respectively) and colony age ($F_{1,44}$=22.37, P<0.0001; $F_{1,44}$=25.06, P<0.0001, respectively) had a significant effect. At 14 dpi (Fig 2.2A), 12-month colonies produced significantly more mites than 6-8-month colonies on their original host. At 28 dpi (Fig 2.2B), in addition to treatment and colony age, the treatment by colony age interaction was also significant ($F_{3,44}$=3.79, P=0.0168). At 28 dpi, both 12-month Settler CL and TAM 112 colonies produced significantly more mites on TAM 112 than 6-8-month colonies.

Byrd (Cmc4) vs Settler CL (no Cmc): Four treatments (Settler CL colonies on Settler CL and Byrd, and Byrd colonies on Settler CL and Byrd) were analyzed separately at 14 and 28 dpi. ANOVA Type III fixed effects (Table 2.5) indicated that at

both 14 and 28 dpi, treatment ($F_{3,33}$=20.17, P<0.0001; $F_{3,33}$=24.27, P<0.0001) and colony age ($F_{1,44}$=75.07, P<0.0001; $F_{1,44}$=19.92, P<0.0001) had a significant effect, while treatment by colony age interaction was not significant. For both the 6-8-month and 12-month colonies produced significantly fewer mites on Byrd compared to Settler CL for both mite colony source, and dpi (Fig 2.3).

Wheat Leaf Curling Rating

ANOVA type I test for fixed effects (Table 2.6) indicated that there were significant treatment and year differences in leaf curling rating, but there was no significant treatment by year interaction. Therefore, data from both years were combined for further analyses. ANOVA Type III fixed effects (Table 2.7) indicated that treatment ($F_{3,33}$=20.17, P<0.0001), dpi ($F_{3,33}$=20.17, P<0.0001), and colony age ($F_{1,44}$=75.07, P<0.0001) had significant effects. The treatment by dpi interaction was close to being statistically significant ($F_{9,328}$=1.79, P=0.0693). Data were analyzed separately for 14 dpi and 28 dpi, and for each resistant varieties in similar manner with mite count data.

For TAM 107, no significant differences were found between all treatments (Fig 2.4). Mean leaf curling rating were higher at 28 dpi (2.4) compared to 14 dpi (1.0). For TAM 112, significant reduction of leaf curling compared to Settler CL were shown at both 14 dpi (0.4 vs 1.2) and 28 dpi (1.1 vs 2.5) (Fig. 2.5). Mean leaf curling rating for TAM 112 significantly increased with longer mite infestation (0.4 vs 1.1). For Byrd, mean leaf rating was significant lower compared to Settler CL at both 14 dpi (0.4 vs 1.2) and 28 dpi (0.8 vs 2.5) (Fig. 2.6). No significant increase was found in leaf curling rating for Byrd at 28 dpi compared to 14 dpi.

Mite Population Genetic Variation

Results from rDNA data found mixed populations of Type 1 and Type 2 WCM in all colonies (Table 2.8). The majority of mites were Type 2 with 83.7% in TAM 112 colony, 80.5% in Byrd colony, 78% in TAM 107 colony, and 76.7% in Settler colony.

Discussion

Plants are thought to have three major resistance categories: antixenosis, antibiosis, and tolerance (Painter, 1951; Smith, 2005). Antixenosis plant traits adversely affect arthropod behavior, leading to reduced colonization or acceptance of a plant as a host. Antibiosis describes adverse effects of resistant plants on herbivore physiology and life histories such as reduced growth, survival, and fecundity. Tolerance is the ability of plants to withstand or compensate for arthropod injury to a degree exceeding susceptible plants. Resistance against WCM has mainly been characterized by antibiosis (reduction of mite population increase) and/or plant tolerance (reduction of leaf curling symptoms) (Harvey et al., 1995b, 1999, 2001; Malik et al., 2003b; Dhakal et al., 2017; Carver et al., 2016; Khalaf et al., 2019; Zhao et al., 2021). However, most of the past studies only addressed these plant traits after short-term (7-14 days) mite infestation periods. This is approximately one to two generations of WCM. In this study, mite populations were allowed to go through multiple generations on resistant varieties to gain a better estimation of their adaptation potential. In addition to mite population counts, leaf curling symptoms of resistant varieties were evaluated after long-term (28 days) mite infestation to gain better estimation of the stability of antibiosis and tolerance traits.

For TAM 107 (*Cmc3*), antibiosis against WCM populations was ineffective. Mite reproduction on TAM 107 was equal to the susceptible variety Settler CL (no *Cmc*). These results are consistent with previous studies that have shown adaptation of mite populations to this gene (Harvey et al., 1997; Khalaf et al., 2019). For TAM 112 (*Cmc3* + *Cmc4*), antibiosis was measurable after mites were held in colony 6-8-month on TAM 112. However, after 12-months, mite reproduction at 28 dpi on TAM 112 was no different compared to Settler CL. Despite having TAM 112 as a parent, Byrd (*Cmc4*) was able to maintain effective antibiosis with mite reproduction reduced by 60-70% compared to Settler CL in all experiments. This indicates that *Cmc4* contributes differently to plant defense responses in TAM 112 and Byrd. Another variety OK05312 (*Cmc4*) was shown to support even higher numbers of mites and leaf curling symptoms compared to Byrd (Carver et al., 2016; Luong et al., unpublished). Future studies are needed to evaluate the expression level and mechanisms of *Cmc4* in different genetic backgrounds. It is important to note that the average mite density on Byrd at 28 dpi was 545 mites for colonies held on Byrd for 6-8-months and 1113 mites for colonies held 12-months. This is still a significant number of mites, especially considering that few mites are necessary for virus transmission. Furthermore, volunteer wheat developed from these resistant varieties can serve as an adequate green bridge host for mites and virus to over-summer and move to infest and infect newly planted wheat in fall.

No reduction in leaf curling (i.e., tolerance) was seen for any mites feeding on TAM 107. Even though the antibiotic effect was different between TAM 112 and Byrd,

leaf curling symptoms were similar for both varieties with significant reduction compared to Settler CL.

Results from these varieties demonstrated that host plant resistance against WCM through antibiosis is not as stable as plant tolerance. While tolerance only involves plant response, both antibiosis and antixenosis create a reciprocal relationship between a plant and a pest. When a genetically diverse pest population is subjected to intense selection pressure in the form of a resistant crop variety, the more virulent individuals within the population will be more likely to survive and interbreed to form an adapted population. Because virulence is a heritable trait, its frequency is likely to increase with each generation. Consequently, the resistant traits in crops will no longer be effective against the majority of individuals in the pest population (adapted individuals). Host plant adaptation arises more frequently with herbivores that reproduce parthenogenetically (e.g., aphids, mites) (Taggar & Arora, 2017). With this type of reproduction and the relatively short generation time, adapted individuals may become abundant within one or two growing seasons. In the case of WCM, this adaptation was evident with the increase in mite reproductive rates of 12-month colonies compared to 6-8-month colonies in all varieties. However, the loss of antibiosis effectiveness was only observed after long-term (28 days) mite infestation. With extended time, extreme population buildup can make accurate population counting more difficult, thus requiring more time and labor. But these efforts have provided valuable insight into the long-term stability and sustainability of mite-resistant varieties.

It is important to note the fecundity of Settler CL colony mites tested on TAM 112 and Byrd also increased at 12-month compared to 6-8-month. This apparent adaptation when not being exposed to the resistant genes raises questions about the cause of this response. Settler CL was selected as a susceptible check based on field and lab observations and due to its popularity in NE (NASS, 2016). Notably, Settler CL is moderately resistant to moderately susceptible to Hessian fly (Hf) (Baenziger et al., 2011). Additional resistance genes in wheat defense responses to Hf (*H13*, *H23*, and H_{WGRC4} (putative)) are also on chromosome 6DS, the same chromosome region contain *Cmc4* (Liu et al., 2005). Therefore, this cluster comprises multiple arthropod resistance genes. In particular, *H13* is a dominant resistance gene expressing a very high and stable level of antibiosis against a wide range of Hf biotypes and geographic populations. These data could potentially provide an explanation for increased reproductive rates for Settler CL colonies over time when tested on varieties with *Cmc* genes. While these complications might be avoided with isogenic wheat varieties, it is very difficult to obtain this in wheat. Future studies are much needed to better understand the mechanisms of *Cmc* genes and perhaps other plant defense genes against WCM.

Under field conditions, the development of 'biotypes' within a population is a significant concern for the long-term effectiveness of host genetic resistance. The term 'biotypes' has been applied to WCM populations based on their responses to different sources of mite resistance and viral transmission ability. However, the use of the term 'biotype' suffers from some problems due to the lack of underlying genetic compositions. In this study, mixed populations were found in all 6-8-month colonies with Type 2 being

the dominant type. These colonies were field-derived from wheat heads and reared on the same wheat varieties they were collected from. Thus, selection and adaptation likely began during the field season and continued in the lab. Even so, the proportion of Type 1 and Type 2 were similar for all varieties. This suggests that there is no correlation between the occurrences of WCM haplotypes (Type 1, Type 2) and mite-resistant wheat varieties (Harvey et al., 2001). However, this interpretation based on the gene-for-gene concept may be oversimplified the mite-wheat interactions. Current WCM genetic characterization is based on amplicon with length less than 2Kb, while total assembly length was estimated at approximately 15.9Mb (Gupta et al., 2019). Moreover, WCM are capable of reproducing parthenogenetically, thus there could be parthenogenetic clones and/or biotypes adapted to different resistance genes within Type 1 and Type 2 haplotypes.

Although it is not possible to completely prevent the adaptation to new host plants, plant tolerance imposes little selection pressure by having minimal adverse effects on mite biology or behavior. Plant tolerance shifts the focus to managing plant stress responses instead of controlling mite populations (Peterson et al., 2017). Therefore, counter-resistance from mites may be less of an issue. The reduction of leaf curling in resistant varieties could potentially help decrease the total number of mites in the field because there would be less favorable niches on the plant. This could result in increased exposure to other abiotic (e.g., wind, rain) and biotic (e.g., thrips) factors not present in our controlled lab experiments. Further studies are needed to evaluate the effect of reduced curling on mite survival and reproduction in the field. Additionally, thick cuticle

or wax depositions on the leaf surface can be physical barriers that hinder WCM from penetrating the epidermis. Different macromolecules (e.g., lignin, cellulose, suberin and callose), small organic molecules (e.g. phenolics), and even inorganic silica particles have been shown to contribute to the reinforcement of leaf cell walls as a result of feeding by herbivores (Fürstenberg-Hägg et al., 2013).

Conclusion

The long-term stability and sustainability of host plant resistance against WCM depends not only in the development of genetically resistant varieties, but also on the management of this germplasm. Continued efforts have been made to identify new mite resistant genes; however, *Cmc4* (including *Cmc1/ Cmc*$_{TAM112}$) is currently the only characterized mite-resistant gene that remain effective (Khalaf et al., 2019). More wheat varieties with *Cmc4* are commercially available and widely grown in the Great Plains. With the history WCM adaptation to *Cmc3*, it is crucial to address the potential for mite adaptation to *Cmc4*. Results from this study indicate that the effectiveness of antibiosis on WCM populations reduced with mite long-term exposure to different resistance genes in wheat. Overcoming of antibiosis in variety TAM 112 (*Cmc3 + Cmc4*) was identified at 28 dpi with 12-month colonies. In contrast, plant tolerance response remained stable with mite adaptation. Thus, future research focusing on plant tolerance traits will most likely pave the way for more stable and sustainable wheat protection practices against WCM.

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Table 2.1: Analysis of variance type I for fixed effects on mite count for treatment and year for 6-8-month and 12-month colonies. (treatments = Settler CL colonies on Settler CL, TAM 107, TAM 112, and Byrd, TAM 107 colonies on TAM 107 and Settler CL, TAM 112 colonies on TAM 112 and Settler CL, Byrd colonies on Byrd and Settler CL, year = 2019, 2020).

Type I Tests of Fixed Effects – 6-8-month colonies				
Effect	**Num DF**	**Den DF**	**F Value**	**Pr > F**
treatment	9	45	3.65	0.0017
year	1	170	9.42	0.0025
year*treatment	9	170	0.86	0.5658

Type I Tests of Fixed Effects – 12-month colonies				
Effect	**Num DF**	**Den DF**	**F Value**	**Pr > F**
treatment	9	45	2.05	0.0548
year	1	170	3.21	0.0752
year*treatment	9	170	1.10	0.3658

Table 2.2: Analysis of variance type III for fixed effects on mite count for treatments, colony age, and dpi. (treatments = Settler CL colonies on Settler CL, TAM 107, TAM 112, and Byrd, TAM 107 colonies on TAM 107 and Settler CL, TAM 112 colonies on TAM 112 and Settler CL, Byrd colonies on Byrd and Settler CL, colony age = 6-8-month and 12-month colonies, dpi = 14, 28).

Type III Tests of Fixed Effects				
Effect	**Num DF**	**Den DF**	**F Value**	**Pr > F**
treatment	9	99	11.46	<.0001
colony age	1	330	181.72	<.0001
colony age*treatment	9	330	1.48	0.1541
dpi	1	330	2799.77	<.0001
treatment*dpi	9	330	1.30	0.2367
colony age*dpi	1	330	4.98	0.0263
colony age*treatment*dpi	9	330	2.26	0.0184

Table 2.3: Analysis of variance type III for fixed effects on mite count for treatments, and colony age for TAM 107 at 14 and 28-day-post-infestation. (treatments = Settler CL colonies on Settler CL, TAM 107, and TAM 107 colonies on TAM 107 and Settler CL, colony age = 6-8-month and 12-month).

Type III Tests of Fixed Effects – 14 day-post-infestation				
Effect	**Num DF**	**Den DF**	**F Value**	**Pr > F**
treatment	3	33	0.81	0.4987
colony age	1	44	35.91	<.0001
treatment*colony age	3	44	0.74	0.5318

Type III Tests of Fixed Effects – 28 day-post-infestation				
Effect	**Num DF**	**Den DF**	**F Value**	**Pr > F**
treatment	3	33	1.57	0.2160
colony age	1	44	26.45	<.0001
treatment*colony age	3	44	3.14	0.0345

Table 2.4: Analysis of variance type III for fixed effects on mite count for treatments, and colony age for TAM 112 at 14 and 28-day-post-infestation. (treatments = Settler CL colonies on Settler CL, TAM 112, and TAM 112 colonies on TAM 112 and Settler CL, colony age = 6-8-month and 12-month).

Type III Tests of Fixed Effects – 14 day-post-infestation				
Effect	**Num DF**	**Den DF**	**F Value**	**Pr > F**
treatment	3	33	10.80	<.0001
colony age	1	44	22.37	<.0001
treatment*colony age	3	44	1.15	0.3409

Type III Tests of Fixed Effects – 28 day-post-infestation				
Effect	**Num DF**	**Den DF**	**F Value**	**Pr > F**
treatment	3	33	9.15	0.0002
colony age	1	44	25.06	<.0001
treatment*colony age	3	44	3.79	0.0168

Table 2.5: Analysis of variance type III for fixed effects on mite count for treatments, and colony age for Byrd at 14 and 28-day-post-infestation. (treatments = Settler CL colonies on Settler CL, Byrd, and Byrd colonies on Byrd and Settler CL, colony age = 6-8-month and 12-month).

Type III Tests of Fixed Effects – 14 day-post-infestation				
Effect	**Num DF**	**Den DF**	**F Value**	**Pr > F**
treatment	3	33	20.17	<.0001
colony age	1	44	75.07	<.0001
treatment*colony age	3	44	1.02	0.3946

Type III Tests of Fixed Effects – 28 day-post-infestation				
Effect	**Num DF**	**Den DF**	**F Value**	**Pr > F**
treatment	3	33	24.27	<.0001
colony age	1	44	19.92	<.0001
treatment*colony age	3	44	0.68	0.5665

Table 2.6: Analysis of variance type I for fixed effects on leaf curling rating for treatment and year for 6-8-month and 12-month colonies. (treatments = Settler CL colonies on Settler CL, TAM 107, TAM 112, and Byrd, TAM 107 colonies on TAM 107 and Settler CL, TAM 112 colonies on TAM 112 and Settler CL, Byrd colonies on Byrd and Settler CL, year = 2019, 2020).

Type I Tests of Fixed Effects – 6-8-month colonies				
Effect	**Num DF**	**Den DF**	**F Value**	**Pr > F**
treatment	9	45	6.66	<.0001
year	1	168	4.60	0.0334
year*treatment	9	168	1.46	0.1685

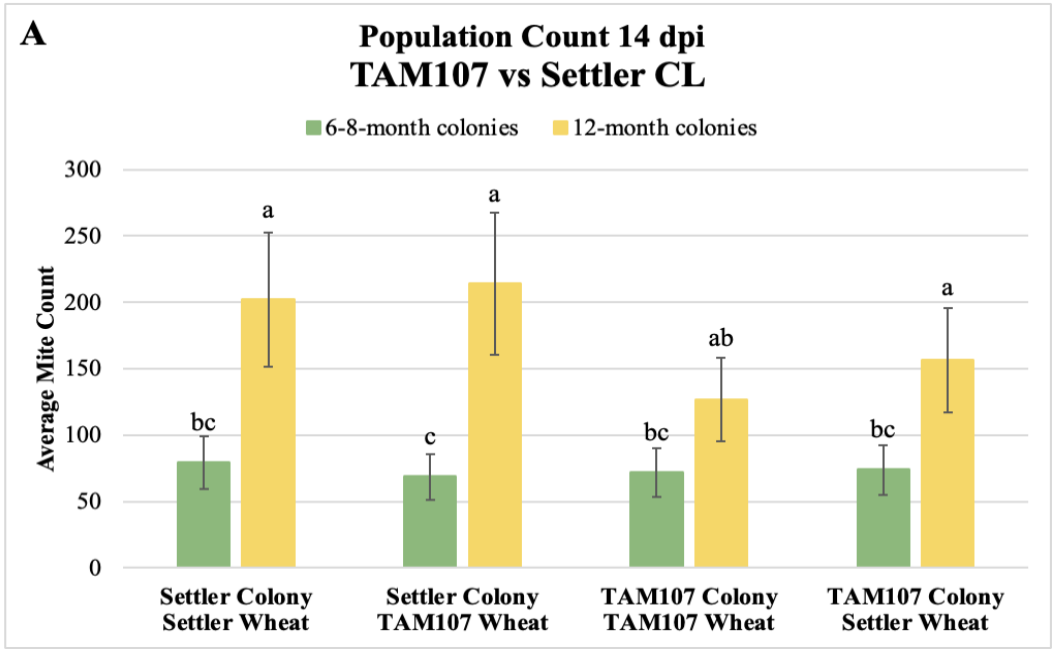
Type I Tests of Fixed Effects – 12-month colonies				
Effect	**Num DF**	**Den DF**	**F Value**	**Pr > F**
treatment	9	45	5.95	<.0001
year	1	168	3.83	0.0520
year*treatment	9	168	0.67	0.7374

Table 2.7: Analysis of variance type III for fixed effects on leaf curling rating for treatment, colony age, and dpi. (treatments = Settler CL colonies on Settler CL, TAM 107, TAM 112, and Byrd, TAM 107 colonies on TAM 107 and Settler CL, TAM 112 colonies on TAM 112 and Settler CL, Byrd colonies on Byrd and Settler CL, colony age = 6-8-month and 12-month, dpi = 14, 28).

Type III Tests of Fixed Effects				
Effect	**Num DF**	**Den DF**	**F Value**	**Pr > F**
treatment	9	99	14.28	<.0001
dpi	1	328	126.71	<.0001
treatment*dpi	9	328	1.79	0.0693
colony age	1	328	47.67	<.0001
colony age*treatment	9	328	0.69	0.7175
colony age*dpi	1	328	5.16	0.0237
colony age*treatment*dpi	9	328	0.30	0.9736

Table 2.8: The positive (successful amplification) PCR samples and the occurrence of WCM Type 1 and Type 2 identified by rDNA in 2019 and 2020 in 6-8-month colonies.

Mite Source	Year	Number of positive PCR samples	Positive PCR samples (%)	Type 1 (%)	Type 2 (%)	Total	
						Type 1	Type 2
Settler Colony	2019	26/48	54.2%	15.4%	84.6%	23.3%	76.7%
	2020	17/30	56.7%	35.3%	64.7%		
TAM 107 Colony	2019	20/24	83.3%	30.0%	70.0%	22.0%	78.0%
	2020	21/24	87.5%	14.3%	85.7%		
TAM 112 Colony	2019	22/24	91.7%	22.7%	77.3%	16.3%	83.7%
	2020	21/24	87.5%	9.5%	90.5%		
Byrd Colony	2019	20/24	83.3%	15.0%	85.0%	19.5%	80.5%
	2020	21/24	87.5%	23.8%	76.2%		



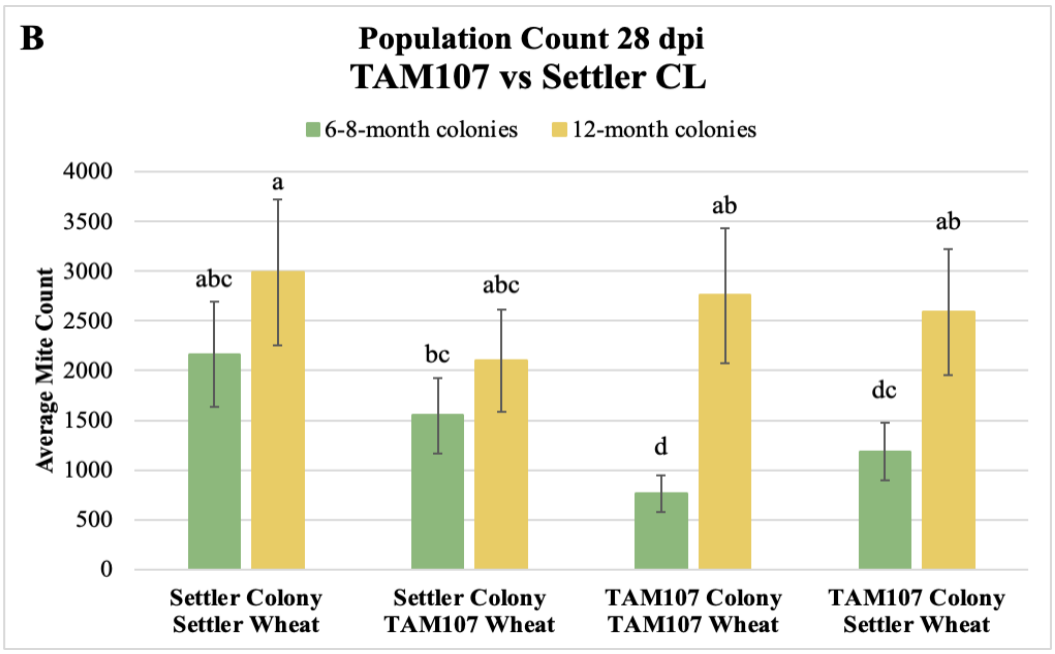


Figure 2.1: Comparisons of average number of WCM per plant after being held on Settler CL (susceptible) and TAM 107 (*Cmc3*) wheat at 14- (A) and 28-day-post-infestation (B). Settler and TAM 107 colonies were field collected and held on those varieties for 6-8 months or 12 months before being tested.

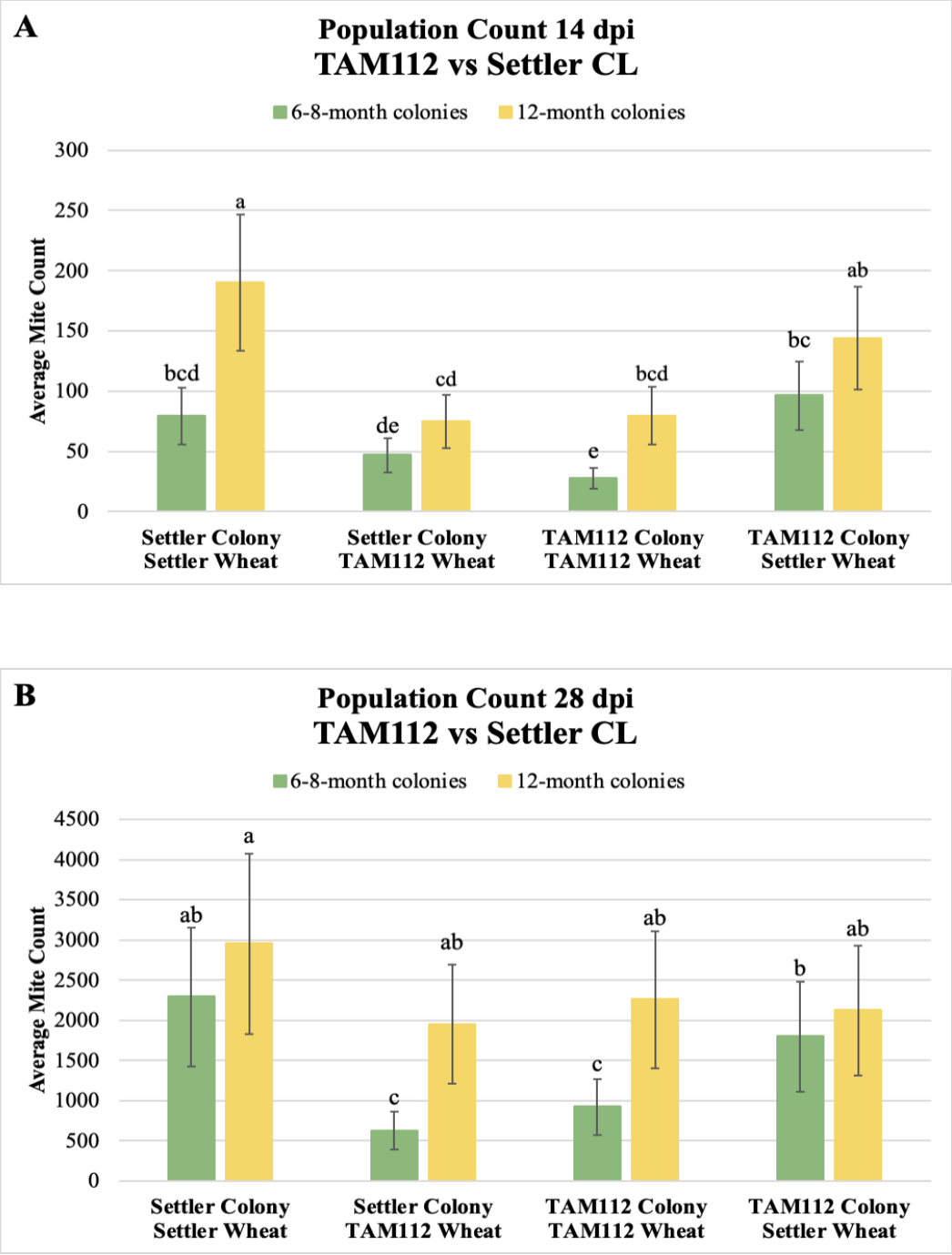


Figure 2.2: Comparisons of average number of WCM per plant after being held on Settler CL (susceptible) and TAM 112 (*Cmc3* + *Cmc4*) wheat at 14- (A) and 28-day-post-infestation (B). Settler and TAM 112 colonies were field collected and held on those varieties for 6-8 months or 12 months before being tested.

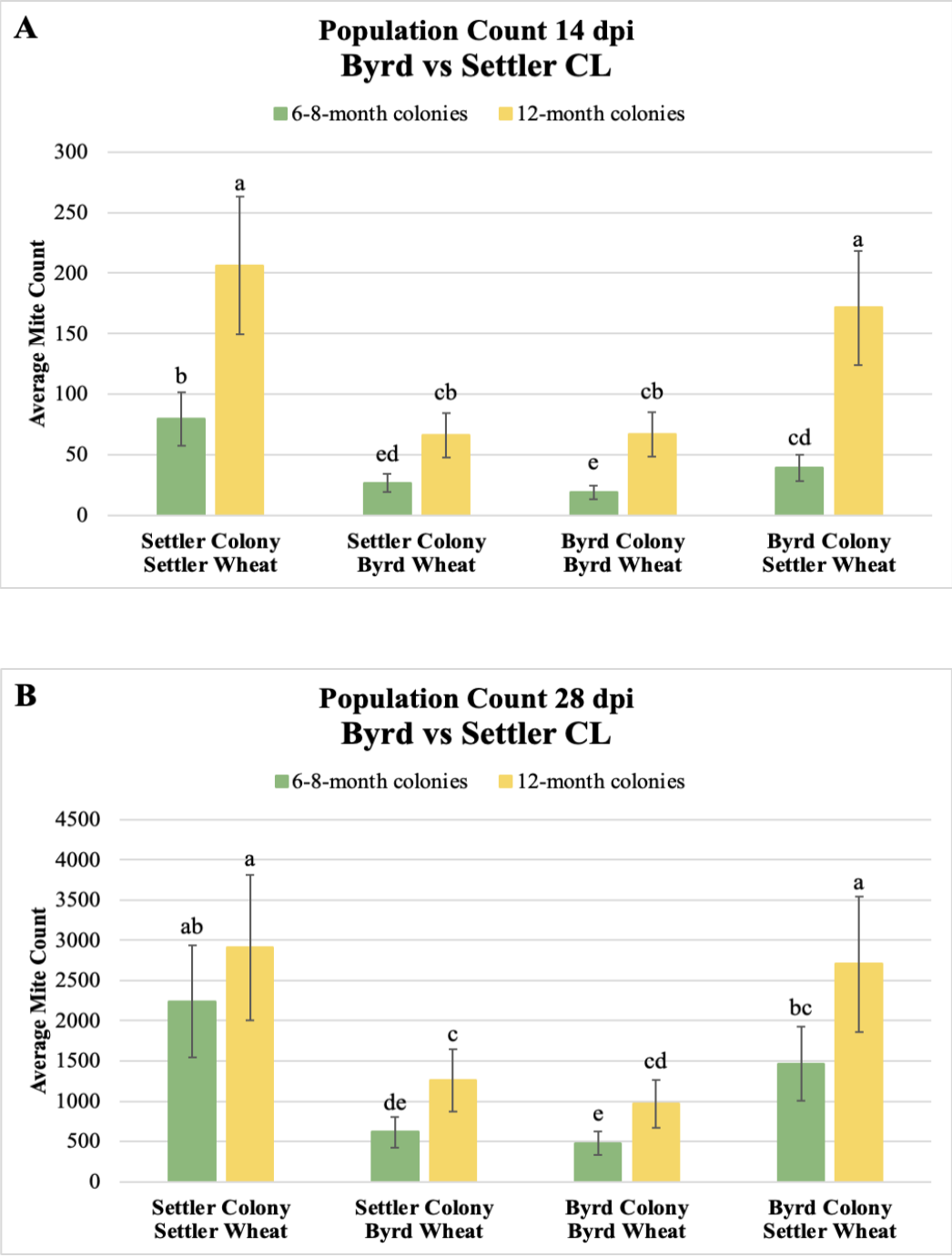


Figure 2.3: Comparisons of average number of WCM per plant after being held on Settler CL (susceptible) and Byrd (*Cmc4*) wheat at 14- (A) and 28-day-post-infestation (B). Settler and Byrd colonies were field collected and held on those varieties for 6-8 months or 12 months before being tested.

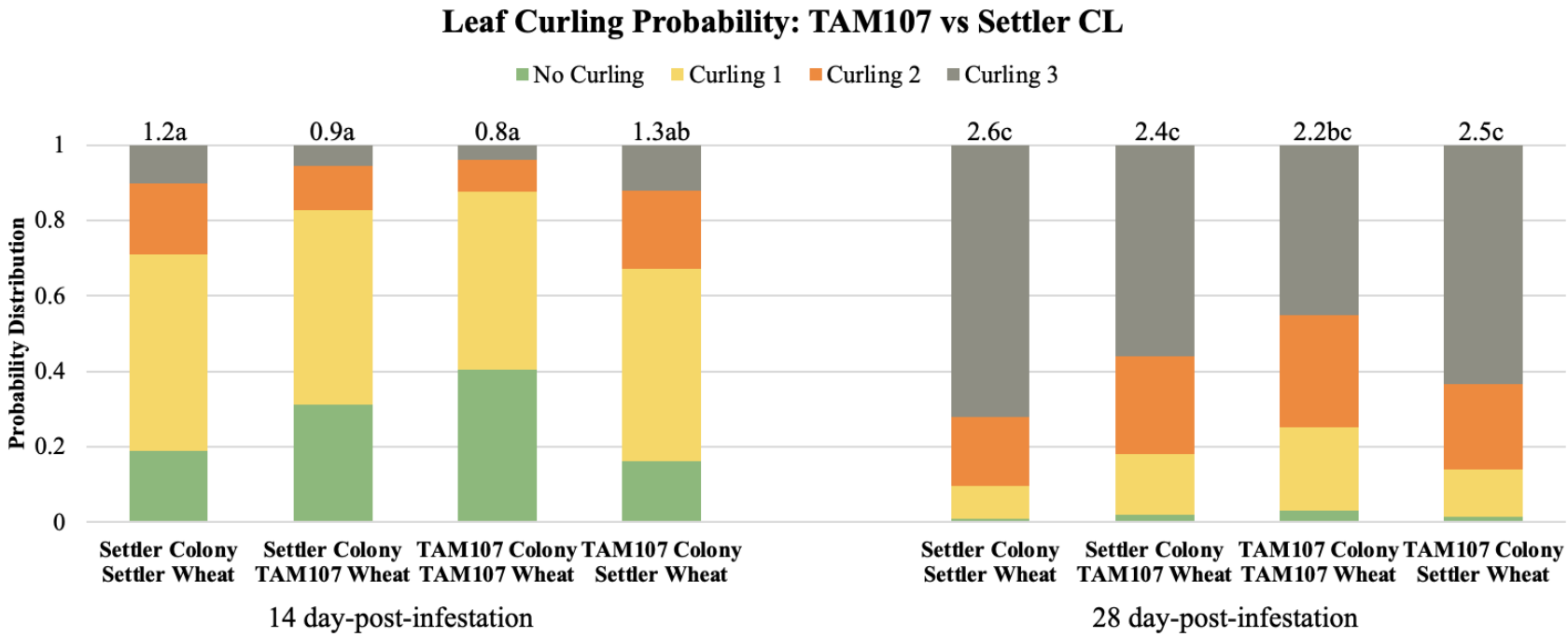


Figure 2.4: Comparisons of leaf curling rating on Settler CL (susceptible) and TAM 107 (*Cmc3*) at 14- and 28-day-post-infestation with Settler CL and TAM 107 colonies.

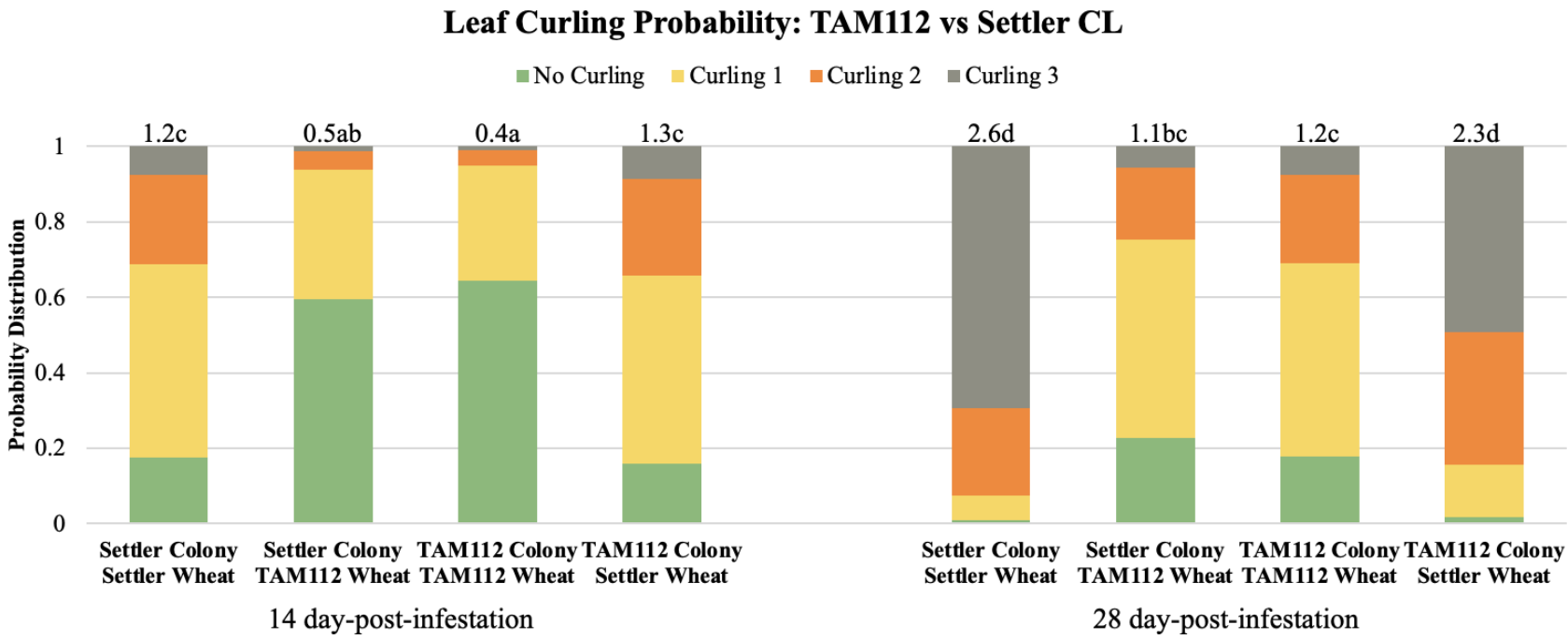


Figure 2.5: Comparisons of leaf curling rating on Settler CL (susceptible) and TAM 112 (*Cmc3* + *Cmc4*) at 14- and 28-day-post-infestation with Settler CL and TAM 112 colonies.

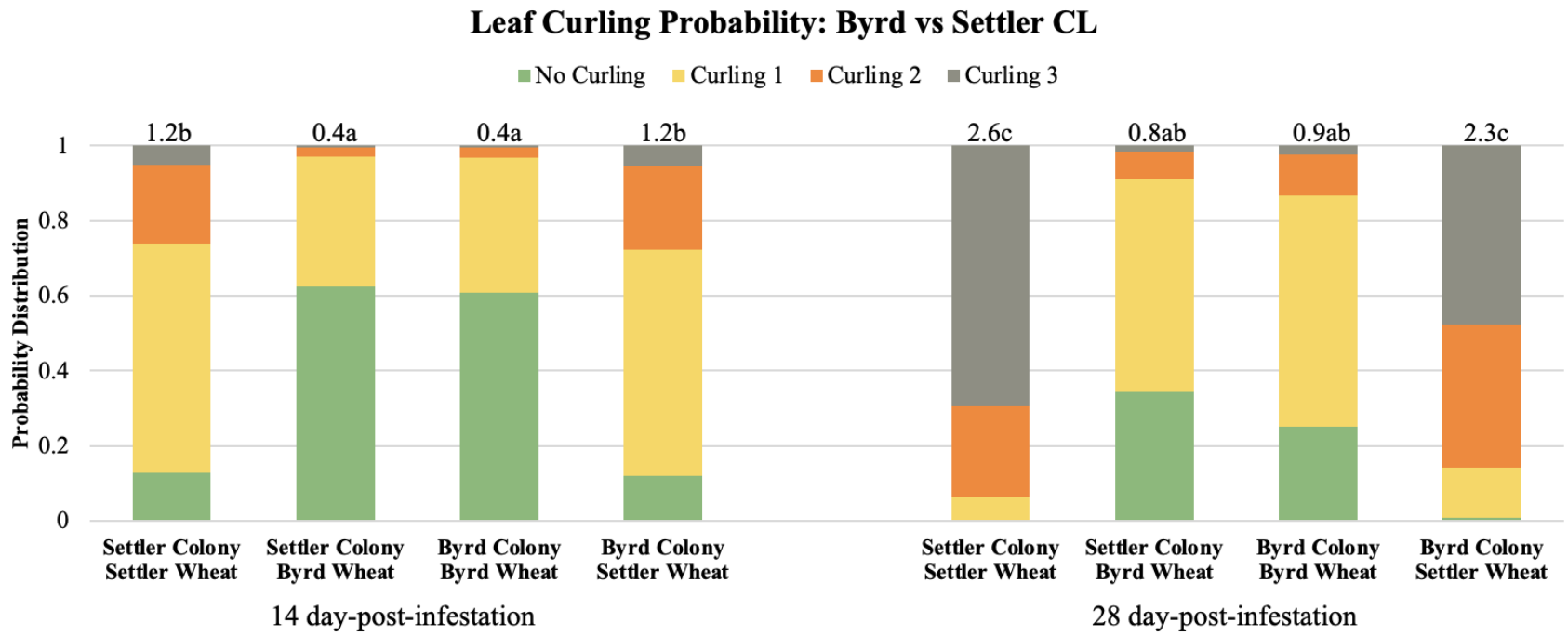


Figure 2.6: Comparisons of leaf curling rating on Settler CL (susceptible) and Byrd (*Cmc4*) at 14- and 28-day-post-infestation with Settler CL and Byrd colonies.

CHAPTER 3

WHEAT TRANSCRIPTOMIC RESPONSES TO LONG-TERM FEEDING BY WHEAT CURL MITES

Introduction

Wheat (*Triticum aestivum* L.) is one of the most crucial crops worldwide, contributing significantly to human food security. Wheat production is affected by many different pests; however, the wheat curl mite (WCM), *Aceria tosichella* Keifer, is one of the most economically significant global pests of wheat. When the microscopic WCM (ca. 0.2 mm long) arrives on a wheat plant, it moves to the base of the newest leaf developing within the whorl and begins feeding (Somsen & Sill, 1970). WCM feeds on the epidermal tissues in the grooves between leaf veins, creating damage to bulliform cells (Styer & Nault, 1996). This feeding impacts the ability of the leaves to unfurl. Mite-infested leaves tend to have their edges curled tightly toward the mid-rib, and the tips of new leaves can become trapped in this rolled leaf forming a loop (Slykhuis, 1955; Somsen & Sill, 1970; Styer & Nault, 1996). The whorl and curled leaves provide WCM a more humid micro-environment beneficial for survival and reproduction, and shelter from miticidal exposure. WCM feeding damages plants by withdrawing the nutrients and distorting leaf growth, thus reducing photosynthesis and respiration (Sabelis & Bruin, 1996). Direct feeding from large populations of WCM can result in ~15% yield loss (Harvey et al., 2000).

The main impact of WCM results from their ability to transmit viruses to wheat. In North America, WCM is the only known vector of *Wheat streak mosaic virus* (Slykhuis, 1955), *High Plains wheat mosaic virus* (Harvey et al., 1997), and *Triticum mosaic virus* (Seifers et al., 2009). While co-infections are common (Byamukama et al., 2014; Mahmood et al., 1998), significant impact on wheat yield and quality can result

from the presence of only one or more viruses in the disease complex (Mahmood et al., 1998; Seifers et al., 2009). WCM reproduces rapidly. With temperature between 23 to 27 ^{o}C, a new generation can develop every 8 to 10 days (Staples & Allington, 1956). Mites disperse via wind currents, increasing their ability to spread viruses. The presence of volunteer wheat plays a significant role in the survival and spread of WCM and the epidemiology of viruses in winter wheat (Somsen & Sill, 1970). Volunteer wheat, especially that emerging before wheat harvest, provides a 'green bridge' to sustain the WCM between summer harvest and the emergence of the new crop in the fall (Wegulo et al., 2008; Wosula et al., 2015). Current management strategies for this wheat-mite-virus complex focus on reducing the impact of 'green bridge' hosts, adjusting planting date, and resistant wheat varieties (McMechan, 2012; McMechan & Hein, 2016).

Understanding the wheat-mite-virus complex is challenging because WCM is a cryptic species complex (Skoracka et al., 2012). In North America, two *Aceria tosichella* haplotypes have been identified (Type 1 and Type 2) based on their genetic differences of mitochondrial DNA cytochrome oxidase I and II (COI and COII) and ribosomal DNA internal transcribed spacer 1 (ITS1) (Hein et al., 2012). Biological differences between these WCM genotypes have been shown for wheat virus transmission efficiencies (McMechan et al., 2014; Seifers et al., 1997; Tatineni et al., 2016; Wosula et al., 2015), reproductive ability on virus-infect plants (Siriwetwiwat, 2006), effects of temperature on population growth rates (Kuczyński et al., 2016), as well as a differential response to several mite resistant genes in wheat (Dhakal et al., 2017; Harvey et al., 1999).

Historically, wheat has not been found to possess significant resistance against the WCM (Harvey & Livers, 1975). This led to efforts to identify and develop resistance genes from close relatives of wheat. To date, four different curl mite colonization (*Cmc*) genes have been identified, chronologically *Cmc3*, *Cmc1*, *Cmc2*, and *Cmc4* (Skoracka et al., 2018). *Cmc3* was translocated from rye (*Secale cereale* L.) to chromosome arm 1AL of wheat and released commercially as 'TAM 107' (Malik et al., 2003; Porter et al., 1987). However, the extensive planting of 'TAM 107' during the 1980's into the mid 1990's led to WCM adaptation and loss of effectiveness of the gene (Harvey et al., 1995; Seifers et al., 1997). *Cmc2* was found in *Agropyron elongatum* (Host) Beauv. and translocated to chromosome arm 6DL of wheat (Whelan & Hart, 1988), but there has been no further development. *Cmc1* was transferred from *Aegilops tauschii* (Coss.) Schmal to chromosome arm 6DS of wheat (Thomas & Conner, 1986; Whelan & Hart, 1988). *Cmc1* is a single dominant resistance gene and was used to develop breeding material with a variety release (Thomas et al., 2012). *Cmc4* was transferred from *Ae. tauschii* and found to also be on the short arm of chromosome 6D in wheat, but despite being on the chromosome 6DS in wheat, *Cmc1* and *Cmc4* were found to be independent (Malik et al., 2003). Mite resistance has been found in the variety 'Byrd' that originated from one of it parents , 'TAM 112' (Carver et al., 2016). Recently, the *Cmc* gene in TAM 112 was mapped in the chromosome 6DS, similar to *Cmc4* (Dhakal et al., 2018). Haplotype analysis using TAM 112 suggests that the *Cmc* gene in TAM 112 and *Cmc*4 are the same gene (Zhao et al., 2021).

WCM resistance has great value in controlling the disease complex in the growing crop, but also by reducing mite buildup in volunteer wheat making up the summer 'green bridge'. Recent development of effective virus resistance genes in wheat (*Wsm1*, *Wsm2*, *Wsm3*) (Graybosch et al., 2009; Tatineni et al., 2016; Zhang et al., 2015) also alter the importance of WCM. As the severe impact of the virus lessens with more virus-resistant wheat, the ability of WCM to build up to a large population becomes more significant (Wosula et al., 2018).

While WCM resistance genes have been growing in number, the interactions between wheat's defense mechanisms and WCM's response and adaptation to these genes is still largely unknown. Kiani et al. (2021) have provided the only study so far to identify potential genes and pathways in defense against WCM herbivory. After 24-hour post infestation (hpi), TAM 112 wheat plants showed modifications in their transcriptomes through the expression of genes involved in jasmonic acid (JA) defense pathways, WKRY transcription factors, antioxidation processes, and pathogen-related responses. However, these genes were unaffected in the WCM-susceptible variety 'Karl 92'.

With evidence of WCM adaptation to *Cmc3* (Harvey et al., 1995, 1997, 1999), the stability and long-term efficacy of these defense mechanisms is a concern. Different WCM haplotypes have varied reactions to different resistance genes (Harvey et al., 1999; Malik et al., 2003). Moreover, the rapid reproductive rate of WCM provides long term advantages to mite populations in overcoming antibiotic-based resistance. For the development and effective deployment of a strategy for these resistance genes, it is

important to know the plant-mite interactions and the categories of resistance involved. The goal of this study is to explore the transcriptome-level responses of wheat varieties with mite-resistant genes to continued mite feeding and the exposure of subsequent mite generations to plant defenses resulting from extended mite infestation. Results from this research will provide further insight into the interactions between resistant wheat varieties and WCM, and propose more effective deployment strategies for this management tactic.

Materials and Methods

WCM population maintenance and infestation

The study was conducted using Type 2 WCM (Hein et al., 2012).The mite colony was maintained on ‘Settler CL’ (NH03614) wheat plants in 15-cm diameter pots with plastic cylindrical cages. The cage had two, 8-cm diameter openings covered with Nitex® screen (80-micron mesh opening; BioQuip Products, Rancho Dominquez, CA) on opposite sides one-third the way from the bottom. The colony was maintained under artificial light with a 14:10 (L:D) photoperiod at 22 - 24 °C. Mites were transferred to new wheat plants in pots every four weeks to maintain the colony.

To perform infestation, only active adults (ca. 190-255 μm) displaying normal movement were used. Mites were transferred with the aid of a dissecting microscope (magnification ca. 30-40X) by using a single human eyelash glued to a wooden dowel to transfer individual mites. Ten mites were selected and released onto a small paper

isosceles triangle (1 cm height). The triangles were then placed into the whorl of 2- to 3-leaf stage (14 days after planting) healthy wheat plants.

Plant materials and samples collection

Two hard red winter wheat varieties (*T. aestivum* L.) were used, Byrd and Settler CL. Byrd is a WCM-resistant wheat variety (Haley et al., 2012) and Settler CL is a WCM-susceptible variety (Baenziger et al., 2011). Seeds were planted individually in cone-tainers (4 cm top diameter and 20 cm length) with standard greenhouse mix. These cone-tainers (Steuwe and Sons Inc., Corvallis, Oregon, USA) were covered with tube cages and kept in the growth chamber with 14:10 (L:D) photoperiod at 25^{o}C and ca. 60% relative humidity. Cages were made from clear cylindrical plastic tubes (5 cm diameter and 50 cm length), vented with three 5-cm diameter openings covered with Nitex® screen.

At 14 days after planting, wheat plants were checked for uniformity in phenotypic growth and health before being used for WCM infestation. The study was conducted as a randomized complete block design with a factorial arrangement of treatments consisting of two wheat varieties (WCM-resistant and WCM-susceptible) and two WCM treatments (infested and non-infested). For each treatment, three replicates were used, and for each treatment, a replicate consisted of three individual wheat plants. At 10 dpi, whorl tissue samples were collected. Tissue sampling consisted of collecting leaf whorl tissue (ca. 3 cm) from each of the three plants per treatment and replicate into a single sample and flash-freezing the tissues in liquid nitrogen.

Nucleic acid extraction and mRNA-seq library construction

Wheat whorl tissues (80-100 mg) were ground using 2010 Geno/Grinder® (SPEX SamplePrep, NJ, USA) for 40 seconds at 1400 strokes min^{-1}. Total RNA was extracted from the homogenized tissue using the kit NucleoSpin miRNA for miRNA and RNA purification (Macherey-Nagel, NucleoSpin miRNA, Mini kit for miRNA and RNA purification, ref 740971.50). Extracted total RNA was quantified through Nanodrop 2000c Spectrophotometer (Thermo Scientific TM). Then, stranded mRNA-seq library construction and sequencing (Illumina) was commissioned to Genewiz (South Plainfield, USA). mRNA-seq libraries were sequenced in 150bp paired-end with 20 million reads on average per library.

Transcriptomic analysis

The quality check of the RNA-seq libraries was performed with FASTQC (Andrews, 2010) and reads with a Phred score lower than 20 and length below 45 base pairs were removed with Trimmomatic v0.39 (Bolger et al., 2014). Then, trimmed reads were mapped on the wheat reference genome v2.1 (https://wheat-urgi.versailles.inra.fr/Seq-Repository/Assemblies) (Zhu et al., 2021) with Tophat2 (Kim et al., 2013) using the following parameters: 2 mismatch (-N 2), 0 splicing mismatch (-m 0). The transcripts' reconstruction was performed with Cufflinks v2.2.1 with the following parameters: quantification against the reference annotation only (-G), multi-read-correct (-u), and frag-bias-correct (-b). The differential expressed gene (DEG)

analysis was performed with Cuffdiff 2.2.1. Differential expressed genes (DEGs) were identified with the following parameters: *P*-values ≤ 5% and false discovery rate (FDR) $|\log_2(\text{Infested/Contol})| \geq \log_2(2)$. All the statistical analysis were performed with R using the packages: stats (Venables & Ripley, 2002) and WGCNA (Langfelder & Horvath, 2008).

Functional annotation

Gene ontology (GO) information was obtained from the IWGCS annotation v1 (https://urgi.versailles.inra.fr/download/iwgsc/IWGSC_RefSeq_Annotations/v1.0/). The GOBU package was used for enrichment calculations (Lin et al., 2006). The full set of wheat gene annotation was used as the reference comparison set against down or upregulated DEGs. The *P*-values were calculated using Fisher's exact test and corrected for multiple testing with the FDR method by using the R module called '*P*-adjust'.

Segmentation/change-point analysis

Segmentation analyses were performed using the R package changepoint v1.0.6 (Killick et al., 2016/2019) with Binary Segmentation method and BIC penalty on the mean change. The gene density was calculated in sliding windows of 10 Mb with a step of 1 Mb.

Data Availability Statement

The raw datasets generated during the sequencing of current study are available on this link:

https://dataview.ncbi.nlm.nih.gov/object/PRJNA765290?reviewer=nc1ea48oagugv3v0ulpq7h8ih

Results

Wheat transcriptomic responses to long-term feeding by WCM

For this study, two wheat varieties were selected because of their susceptibility (Settler CL) or resistance (Byrd) to WCM. Twenty days post infestation (dpi), wheat leaves experienced a different morphology for each variety. Leaf curling was observed for the susceptible variety (Figure 3.1A), while the leaves of the resistant variety remained flat (Figure 1B). To further investigate the underlying mechanisms of wheat responses against WCM, transcriptomic profiles of WCM-infested and -uninfested control plants was performed at 10 dpi.

The sequencing of the RNA-seq libraries for the 2 varieties at 10 dpi (WCM infested and uninfested control) generated 20.7 million paired-end reads on average (Supplemental Table 3.1). Reads were mapped on the reference genome v2.1 of the variety Chinese Spring, (Zhu et al., 2021) and an average of 17.2 million paired-end reads (83%) were mapped on the reference genome assembly (Supplemental Table 3.1). The PCA analysis was run for the 106,817 genes expressed in at least one condition, and responses for the two varieties separated in different groups (PC1, 31.2%) (Figure 3.2).

However, there was not a clear distinction between the infested or control conditions (PC2, 23%) (Figure 3.2).

The number of differentially expressed genes (DEGs) were characterized for the following comparisons: Byrd control *vs.* Byrd infested, Byrd control *vs.* Settler CL control, Byrd control *vs.* Settler CL infested, Byrd infested *vs.* Settler CL control, Byrd infested *vs.* Settler CL infested and Settler CL control *vs.* Settler CL infested, with the following parameters: |FC|>2 and *P*-value < 5% (Supplemental Table 3.2). In total 11,016 non-redundant DEGs were identified. The number of genes up or downregulated for each comparison is shown in Figure 3.3A. Among the 1,822 DEGs in the resistant genotype, 75.5% (1,376 genes) were upregulated at 10 dpi. By comparison, 2,611 genes were differentially expressed in the susceptible genotype, including 31.7% (828 genes) upregulated genes at 10 dpi (Figure 3.3A). Comparing the uninfested condition of the two varieties, 4,322 genes were differentially expressed with 67.5% (2,917 genes) upregulated genes in Settler CL. After infestation, 5,717 genes were differentially expressed between both varieties and 55.2% (3,154 genes) of the genes were upregulated in Byrd.

The overlap of the 4,084 DEGs up or downregulated for the Byrd and Settler CL comparisons of control and infested conditions was represented with a Venn diagram (Figure 3.3B). One hundred seventy-one genes were commonly upregulated after WCM infestation and 134 were commonly differentially expressed between the conditions upregulated in the resistant variety and downregulated in the susceptible variety after WCM infestation (Figure 3.3B).

The function of the DEGs was impacted differently for each variety. In the resistant variety, lipid transport, lipid localization, or sugar metabolic process functions were downregulated (Supplemental Table 3.3). Alternatively, upregulated genes in the resistant variety were related to immune response, immune system process, and regulation of defense response functions (Supplemental Table 3.3). On the other hand, downregulated genes in the susceptible variety were related to positive regulator of stomatal complex development, tissue development, plant epidermis development, or polysaccharide catabolic process functions (Supplemental Table 3.3). Upregulated genes of the susceptible cultivar were related to metal ion transport, cellular localization, or defense functions (Supplemental Table 3.3).

Downregulation of the genes located on the telomeric part of the chromosome 3DL of the susceptible wheat variety

Hexaploid wheat is composed of three sub-genomes: A, B and D. Version 2.1 of the annotation, which was used in our analysis, displayed the repartition of the protein coding genes equally among the 3 sub-genomes, A: 35,345 genes, B: 35,643 genes, and D: 34,212 genes (Zhu et al., 2021). Here, we further observed the repartition on the 21 wheat chromosomes of the 4,084 DEGs in Byrd and Settler CL infested plants and their respective control (described in Figure 3.3B). The highest number of DEGs were located on chromosome 3D (501 DEGs) (Supplemental Figure 3.1A).

Among the 501 DEGs located on the chromosome 3D, 381 (76%) genes were downregulated in Settler CL after WCM infestation (Figure 3.4A). Of these 381 genes on

the chromosome 3D, 333 genes were located in the telomeric part of the large arm of the chromosome 3D (Figure 3.4B). Gene functions of these 333 genes were linked to protein N-Linked glycosylation, phytochromobilin metabolic and biosynthesis processes, and positive regulation of stomatal complex. Among the top 10 genes with the highest fold-change, two genes were not expressed in the infested condition of Settler CL and expressed in the uninfested conditions: *TraesCS3D03G0974000LC* (Protein FAR1-RELATED SEQUENCE 5) and *TraesCS3D03G1005800LC* (60S ribosomal protein L5) (Supplemental Table 3.2). Other genes had functions related to GDSL esterase/lipase, Proline-rich protein, Germin-like protein 1-1, Dirigent protein, Arginine decarboxylase, or Oxidation resistance protein 1 (Supplemental Table 3.2).

Hierarchical clustering exhibited gene function enrichment specific for each wheat variety

Overall, 11,016 DEGs displayed up or downregulated between all the conditions. Clustering patterns of DEGs under WCM infestation were determined by hierarchical clustering analysis of all DEGs. The 11,016 DEGs were grouped into 11 clusters that included from 51 (cluster 10) to 4,030 (cluster 1) genes (Figure 3.5). Clustering analysis showed genes activated in Settler CL infested and uninfested conditions (cluster 1) were related to lipid transport and localization and protein methylation and alkylation (Supplemental Table 3.4). Cluster 6 contained genes activated in Byrd infested and uninfested conditions with functions related to protein transport and localization, and maintenance of cellular protein location. Genes with functions related to asparagine

synthase, catalytic activity, protein dimerization or ligase activity were part of the cluster 8, where genes were activated in Byrd after WCM infestation (Supplemental Table 3.4).

Variety-specific metabolic pathway response to WCM infestation

We used Mapman to investigate the variation of the metabolism pathways and processes in both cultivars in response to long-term feeding by WCM. Our results indicate that more pathways related to cell wall, secondary metabolites, redox states, or hormonal pathways (e.g., JA and ABA) were detected in the susceptible cultivar at 10 dpi (Figure 3.6A). This could explain the higher number of DEGs observed for the susceptible variety. However, genes involved in these pathways were mainly downregulated in the susceptible variety (Figure 3.6A). The number of genes related to metabolic pathways in the resistant cultivar were low, but these genes were mostly upregulated (Figure 3.6A). Further, upregulated DEGs in the resistant variety were involved in hormonal pathways such as JA and ABA, redox state, or cell wall biosynthesis (Figure 3.6B).

Discussion

Early and late defense signaling mechanisms contribute to a robust defense against insect attack (Howe & Jander, 2008; Maffei et al., 2007; Nalam et al., 2019). Leaf curling resulting from WCM feeding has been used to score wheat varieties as susceptible or resistant against WCM (Dhakal et al., 2017; Zhao et al., 2021). At 20 dpi, visual differences were detectable between leaves of susceptible and resistant varieties.

Susceptible plants displayed longitudinal leaf curling and leaves trapped within the curl of older leaves (Figure 3.1). However, symptomatic leaves are not easily noticeable until leaves were highly infested with WCM. Leaf rolling or curling has been described in crops leaves in response to various stresses such as salt, drought, and WCM (Kadioglu et al., 2012; Skoracka et al., 2018). A few genes have been involved in these leaf morphological changes such as, *LCR* (*LEAF CURLING RESPONSE*) (Song et al., 2012), TaDUFF699 gene family (Zhou et al., 2020), *OsLBD3-7*, *NLR1*, and *ACL1* (Myśków et al., 2018). These previous studies demonstrated the modification of the plant transcriptome linked to changes in leaf morphology. The reduction of curling symptoms in resistant varieties could potentially help decrease the total number of WCM in the field due a less favorable niche on the plant and increased exposure to other abiotic and biotic factors not present in our controlled lab experiments.

Wheat transcriptome responses to short-term feeding (1 dpi) by WCM has been recently reported and provided insights about early defense mechanisms utilized by wheat against WCM (Kiani et al., 2021). However, WCM has a rapid reproductive rate, with egg-adult developmental times of 7 to 10 days (Slykhuis, 1955; Staples & Allington, 1956). Thus at 10 dpi, the plant will be interacting with the next generation of WCM. While symptoms on wheat leaves are not strong at 10 dpi, the impact of WCM feeding that leads to curling had been started, thus we investigated the variation of the transcriptomic response between resistant and susceptible wheat varieties after long-term feeding (i.e., 10 dpi).

Compared to short-term (1 dpi) feeding by WCM (Kiani et al., 2021), the number of DEGs for each cultivar between infested and uninfested conditions were higher after 10 dpi. Our results showed the proportion of upregulated DEGs were higher in the resistant cultivar only. At 10 dpi, we also observed a higher number of downregulated genes in the susceptible cultivar compared to the susceptible cultivar at 1 dpi as seen by Kiani et al. (2021). These results suggest that defense mechanisms activated early during WCM infestation did not last. At 10 dpi, 134 genes were upregulated in the resistance cultivar and downregulated in the susceptible cultivar. The functions related to these genes showed the importance of the production of stress-related hormones and structural components in biological membranes. Phospholipase A1 genes catalyzes the hydrolysis of fatty acids and the release of alpha-linolenic acid, which has been described as a JA precursor (Canonne et al., 2011). The role of fatty acids in plant defense has been characterized for the response to fungal and insect infections (Walley et al., 2013). Fatty acid levels will increase insect elicitor induced defense response (Li et al., 2016). Fatty acids also have a role in wax composition which represent a physical barrier for insect/pest feeding (Ali et al., 2021; Sharma et al., 2018; Wang et al., 2020). In our experiment, after 10 days, WCM affected leaf morphology by preventing the leaves from unfolding and by consequence proper leaf development. The alteration of the leaf morphology in the susceptible cultivar at 10 dpi affects stomatal development, which could lead to the alteration of the photosynthesis and plant development. The action of WCM on resistant cultivar at 10 dpi was related to primary nitrogen metabolism with the inactivation of asparagine synthase genes that play an important role in nitrogen

assimilation and distribution. Nitrogen also plays a role in gene transcription by its involvement in RNA synthesis (Oliver & McLaughlin, 1977). Collectively, our data suggest that the wheat transcriptome was impacted at 10 dpi in the susceptible variety.

Plant resistance can be separated into three resistance categories: antibiosis, antixenosis, and tolerance (Painter, 1951). Tolerance is the plant's ability to withstand or recover from insect/pest damage; however, the mechanisms underlying tolerance are poorly understood. Recent studies have suggested that phytohormones play a major role in plant tolerance to insects (Chapman et al., 2018; Grover et al., 2020; Onaga & Wydra, 2016). Previously, it was shown that metabolite levels were altered in wheat plants after short-term (1 dpi) feeding by WCM (Kiani et al., 2021; Reddy et al., 2013). While there were many differences in the responses between susceptible and resistant varieties, comparing DEGs between 1 dpi (Kiani et al., 2021) and 10 dpi highlight some important mechanisms that can contribute to strengthening host plant resistance. First, we observed that DEGs related to cell wall composition in the resistant variety were downregulated after 1 dpi (Kiani et al., 2021), but they were upregulated after 10 dpi. This indicates that resistant varieties are able to maintain a cell wall structure after prolonged WCM feeding. Second, phytohormones play key roles in herbivore-induced defenses by activating key early signal transduction pathways (Erb et al., 2012). Our study identified a high number of DEGs involved in JA and ABA that can potentially modulate WCM-induced stress responses. ABA is a phytohormone that regulates plant growth and development, and abiotic stress responses in plants (Fujita et al., 2006). ABA did not show significant induction at 1 dpi in either susceptible or resistant varieties. In contrast, genes related to

ABA were downregulated in susceptible varieties but upregulated in resistant varieties at 10 dpi. We hypothesize that ABA will accumulate in the resistant wheat in response to damage of bulliform cells and photosynthesis and respiration stress from early symptoms of leaf curling. JA also plays an important role in plant response to biotic stress. DEGs related to JA in susceptible varieties did not show significant response at 1 dpi yet they had a mix of both up- and downregulation at 10 dpi. However, JA was upregulated in resistant varieties at both time points. This suggests that JA plays a major role in wheat defense against WCM.

Chemical defenses play a decisive role in induced defense mechanisms against herbivore infestation (War et al., 2012). We saw downregulation of genes related to secondary metabolites in the susceptible wheat variety, but a clear pattern for the resistant variety was not seen at 10 dpi. Interestingly, DEGs related to secondary metabolites were upregulated at 1 dpi in the susceptible variety (Kiani et al., 2021). Five DEGs at 10 dpi had functions related to chymotrypsin inhibitor. Trypsin and chymotrypsin are the major digestive serine proteases in lepidopteran insects. In Arabidopsis, transgenic expression of barley protease inhibitor genes provided enhanced resistance to spider mites (*T. urticae*) (Santamaria et al., 2012). No investigations had been yet performed on the gut composition of WCM feeding on wheat. However, our results suggest a role of chymotrypsin inhibitor in the wheat resistant cultivar at 10 dpi, possibly by countering WCM gut/saliva secretion. Together, these results highlight the defense mechanisms used by the resistant wheat cultivar to limit WCM colonization. Plant resistance against insect herbivory has focused on antibiosis, but evolution and adaptation of target pest

population is inevitable. Focusing on mechanisms that contribute to plant tolerance would be a more sustainable strategy. We believe further studies can benefit from exploring the genetics of morphological features of tolerance (i.e., reduction of curling symptoms) and physiological mechanisms (e.g., ABA affecting photosynthetic rate, growth rate post infestation).

Because of its large genome size (17 Gb), wheat gene space organization was characterized with high gene density in the telomeric chromosome area (International Wheat Genome Sequencing Consortium (IWGSC), 2014; Pingault et al., 2015). The investigation of the location of the downregulated genes in the susceptible cultivar were only found enriched in the telomeric area of the large arm of the chromosome 3D. *Aegilops tauschii* has been identified as a donor of the D-genome for the allohexaploid wheat (*Triticum aestivum* L.) (McFadden & Sears, 1946), and the D sub-genome contains fewer genes than the A and B sub-genomes. Nevertheless, the D-genome has been identified as a reservoir for biotic and abiotic stress tolerance, and *A. tauschii* has been used to transfer useful genes to the allohexaploid wheat by direct hybridization or synthetic wheat for pest/pathogens resistance, abiotic stresses, and quality traits (Assefa & Fehrmann, 2004; Trudgill, 1986). The geographical origin of *A. tauschii* in arid and semi-arid areas has been linked with the drought resistance role of the genes carried by the D-genome. The morphological changes of the leaves in response to drought stress is similar to the response to WCM for wheat. This could result from the inactivation of genes located in the sub-genome D. In this study, downregulated genes were related to stomatal complex development, tissue development, and phytochromobilin. These

functions in leaves are responses to drought for water retention (reduction in stomata density, low transpiration efficiency, and increased stomata size) (Hepworth et al., 2015). Byrd resulted from the crossing of C0970547-7 and TAM 112 (Haley et al., 2012; Rudd et al., 2014). The crossing history of TAM 112 included a *A. tauschii* line, TA2460, known to carry the leaf rust resistance gene *Lr41* and origin of the *Cmc4* gene (Malik et al., 2003). These information attest of the importance of the D-subgenome in resistance to WCM. The investigation of the function of all the genes located in the 3DL telomeric region revealed functions related to plant defense mechanisms. Interestingly, the gene set located on 3DL and downregulated in the susceptible cultivar are not differentially expressed in the resistant cultivar at 10 dpi. Further investigation will be necessary to evaluate the transcriptomic activity of these genes during a time course in the wheat resistant cultivar.

Conclusions

In this study, we provide evidence of defense mechanisms used by a resistant wheat variety containing the *Cmc4* gene against WCM after long-term feeding. Action of phytohormones, combined with lipid signaling and membrane integrity play a role in response to WCM after 10 dpi. A higher number of molecular functions are activated at 10 dpi compared to 1 dpi (Kiani et al., 2021) in the resistant variety. In addition, the importance of the genes located in the sub-genome D of the wheat in response to mite feeding is identified.

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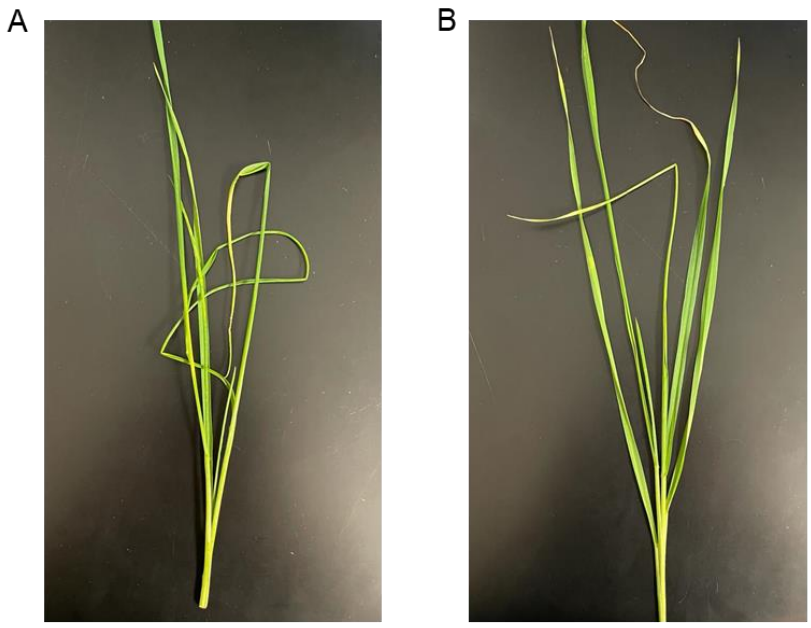


Figure 3.1: Leaf curling resulting from WCM feeding at 20 dpi in Settler CL (A) and Byrd (B). The picture was taken by Tran Kim Ngan Luong.

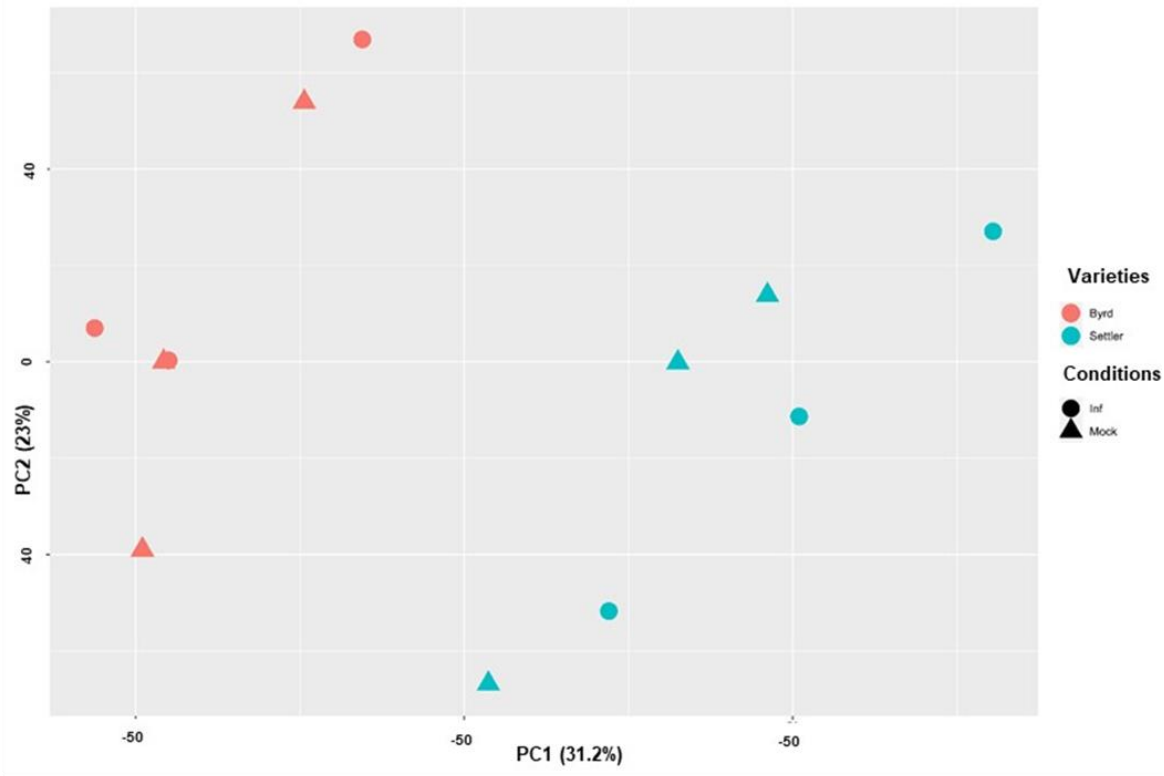


Figure 3.2: PCA analysis of the 106,817 high confidence genes expressed in at least one condition. Wheat cultivar is represented with different colors (red= Byrd and blue= Settler CL) and treatment condition with different shapes (circle=Infested and triangle=control).

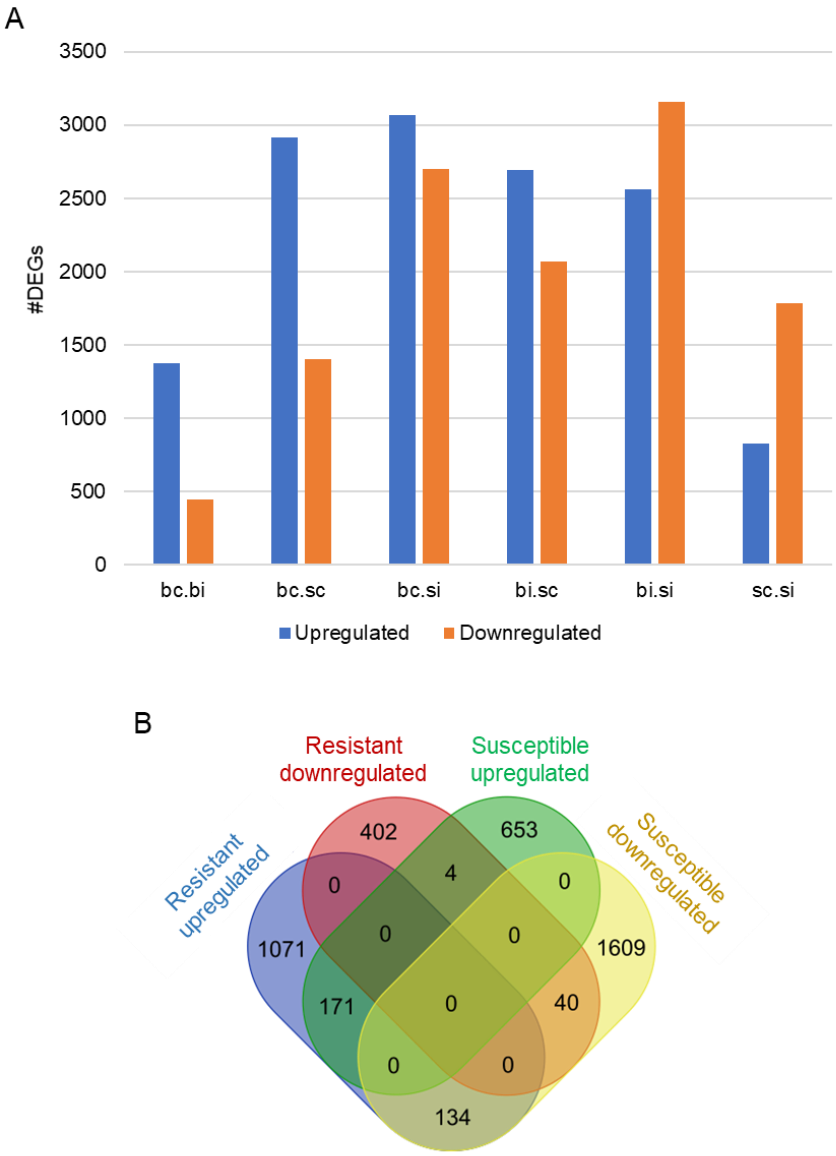


Figure 3.3: Overview of the 11,016 DEGs. (A) Partitioning of the DEGs as up or downregulated for all the comparisons. Bc = Byrd control, bi = Byrd infested, sc = Settler CL control and si = Settler CL infested. (B) Venn diagram representing the overlap of the up and downregulated DEGs between all the comparisons.

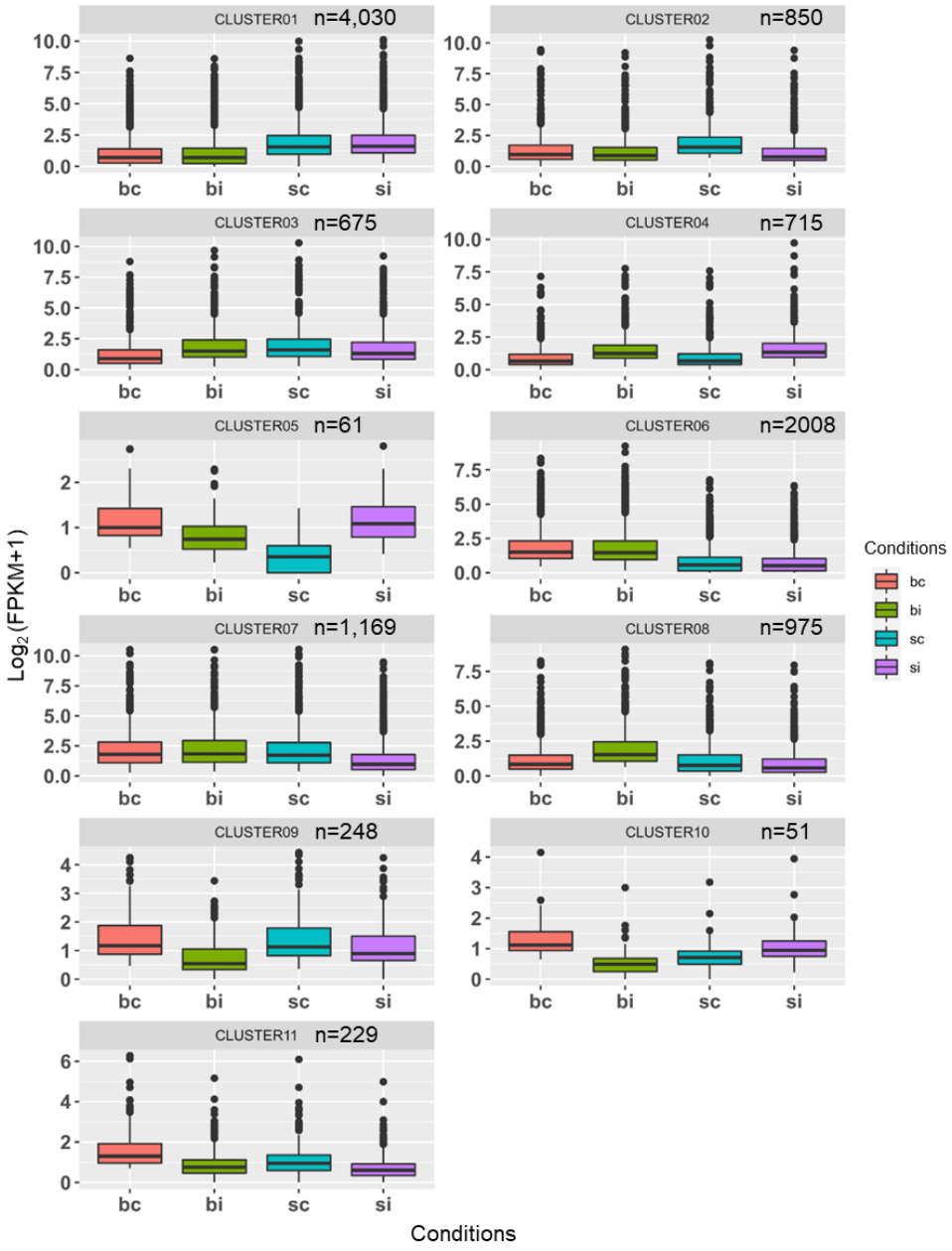


Figure 3.4: DEGs space organization. (A) Repartition of the 4,084 DEGs on the 21 wheat chromosomes. Bc = Byrd control, bi = Byrd infested, sc= Settler CL control and si = Settler CL infested. (B) Plot density of the DEGs on the chromosome 3D. Gene density was represented in a window of 10 Mb with a sliding window of 1 Mb. n indicates the number of genes. Blue dash lines separate the different segments identified with changepoint.

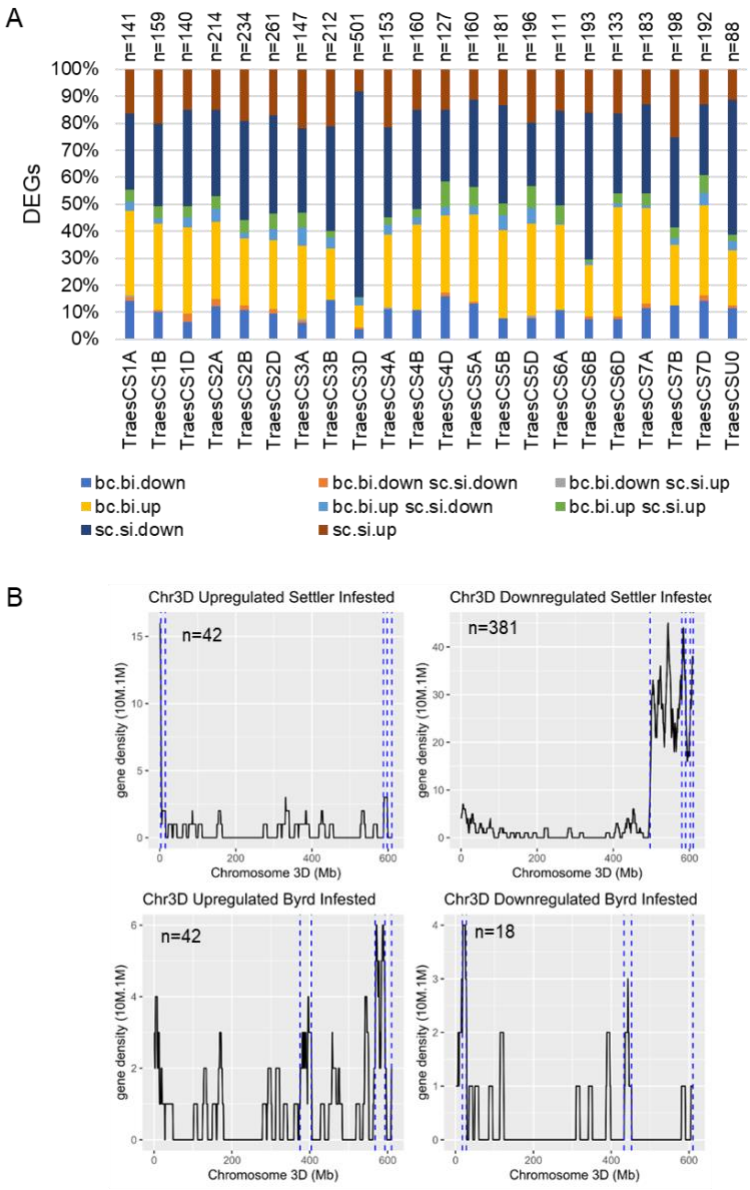


Figure 3.5: Expression profiles of the DEGs for the 11 clusters. Expression values are given in log2 (FPKM+1) (red=bc, green=bi, turquoise=sc and purple=si). Bc = Byrd control, bi = Byrd infested, sc = Settler CL control and si = Settler CL infested.

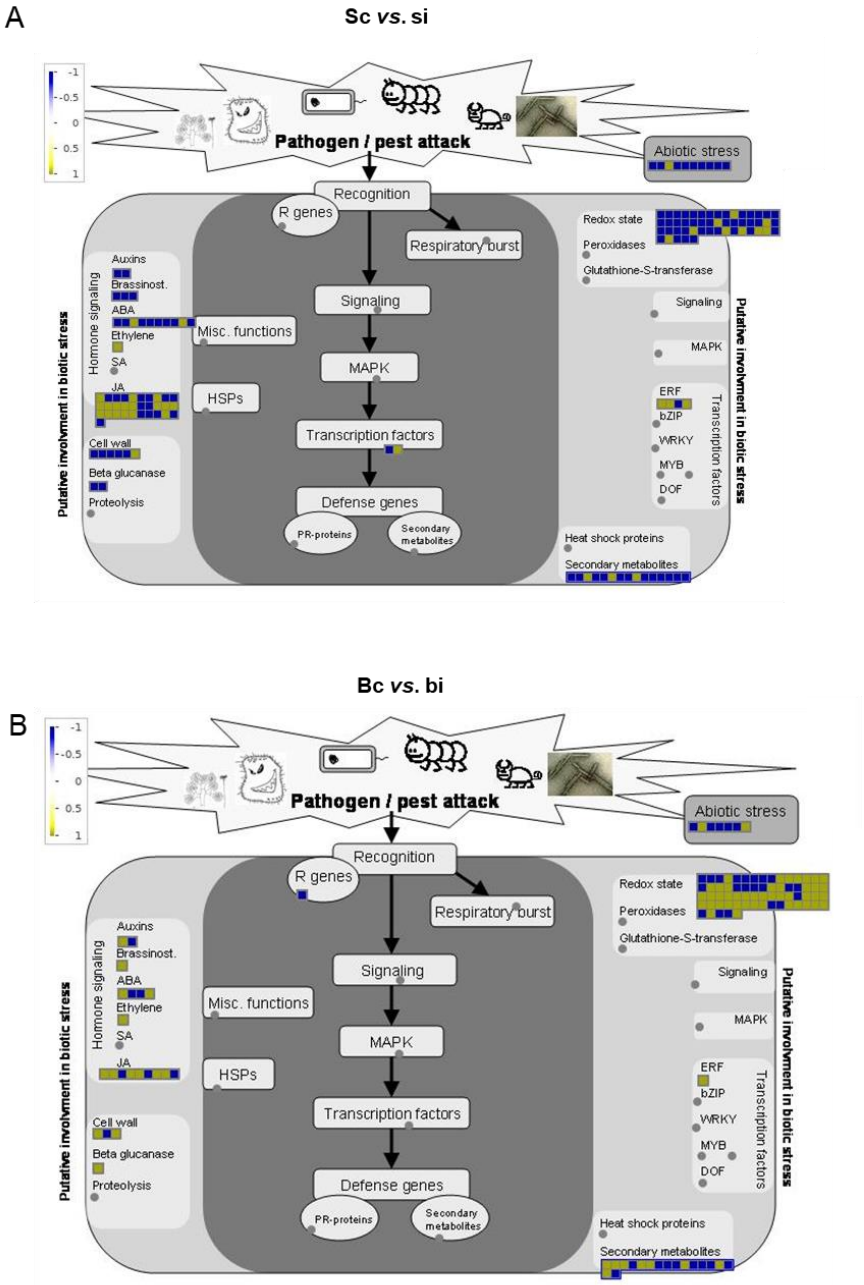
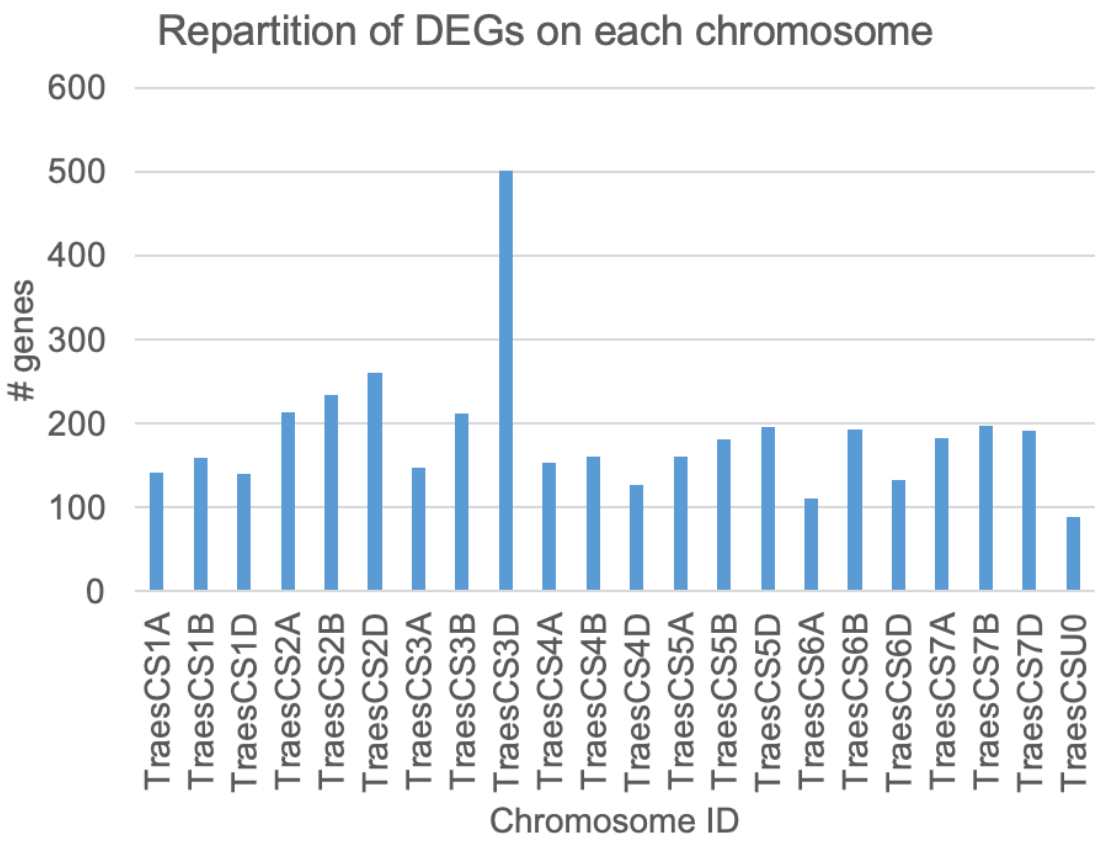


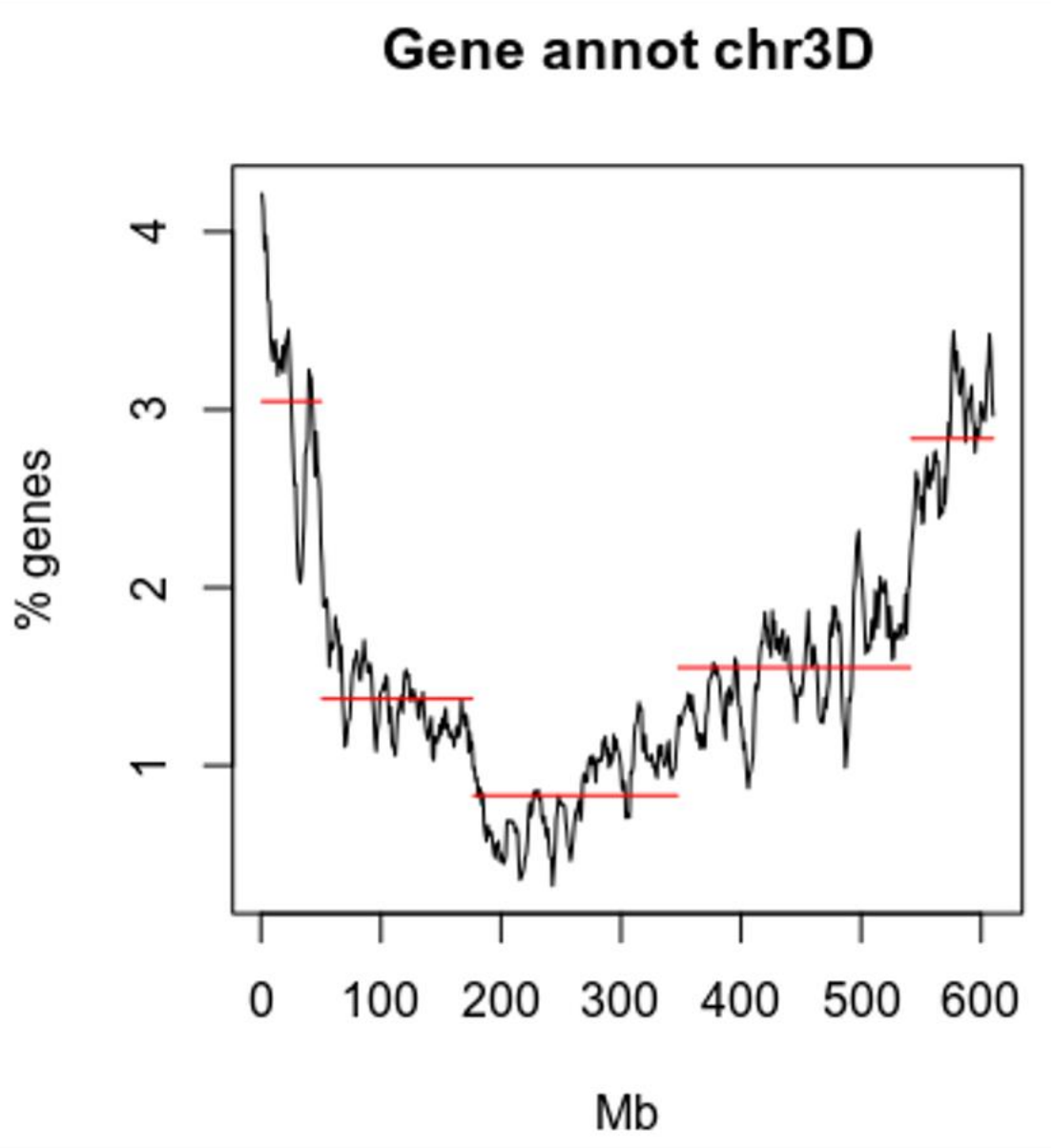
Figure 3.6: Overview of the gene transcriptomic response after WCM infestation using Mapman for the resistant cultivar (A) and susceptible cultivar (B). Each box represents the –log10 (FC). Yellow indicates upregulated gene expression and blue downregulated gene expression in response to WCM. Bc = Byrd control, bi = Byrd infested, sc = Settler CL control and si = Settler CL infested.

Supplementary Information

Supplemental Figures



Supplemental Figure 3.1: Repartition of the 4,084 DEGs on the 21 wheat chromosomes. U0 indicates the scaffolds.



Supplemental Figure 3.2: Gene density of the annotation genes of the chromosome 3D. Red lines represent the segmentation.

APPENDIX 1

SAS CODE FOR MITE COUNT AND LEAF CURLING RATING ANALYSIS

```
data fieldmite;
input year 1-4 trial 6 rep 8-9 treatment 11-12 mite $14-15 wheat
$17-18 dpi 20-21 curling_rating 23 count 25-28;
Datalines;
2019  1     01    01    SL    SL    14    1     66
2019  1     01    03    SL    T2    14    1     47
...
;
run;

/*ANOVA for main effects and interaction of year and treatments*/
title "year*treatment mite count trial 1";
proc glimmix data = fieldmite;
 where colony_age =1;
 class year rep treatment dpi;
 model count = treatment|year / solution dist=negbinomial
htype=1;
 random intercept rep rep*treatment;
 nloptions maxiter = 1000;
 run;

/*ANOVA for main effects and interaction of treatments, colony
age, and dpi*/
title "mite count";
proc glimmix data = fieldmite;
 class colony_age rep treatment dpi;
 model count = treatment|colony_age|dpi / solution
dist=negbinomial;
 random intercept rep rep*treatment/ subject=year;
 lsmeans treatment*colony_age / slicediff = colony_age plot =
meanplot(sliceby = treatment join) adjust = tukey lines ilink;
 nloptions maxiter = 1000;
 run;

/*Mite Count Analysis*/
title 'Mite count analysis - Byrd vs Settler CL - 14dpi';
```

```
proc glimmix data = byrd;
 where dpi= 14;
 class year rep treatment trial;
 model count = treatment|trial / solution dist=negbinomial;
 random intercept rep rep*treatment/subject= year ;
 lsmeans treatment*trial / slicediff = trial plot =
meanplot(sliceby = treatment join) adjust = tukey lines ilink;
 lsmestimate treatment*trial
'SL mite on SL - 14d - trial 1 vs 2' [1, 1 1] [-1, 1 2],
...
/ adjust =  bon exp;
 nloptions maxiter = 1000;
 run;

/*Leaf Curling Analysis*/
 title 'Curling Rating Analysis - Byrd vs Settler CL';
proc glimmix data = byrd initglm;
 class year trial rep treatment dpi curling_rating;
 model curling_rating = treatment | dpi / d = multinomial link =
clogit oddsratio solution;
 random intercept rep rep*treatment/ subject=year;
 nloptions maxiter = 1000;
 store curlingbyrd;
run;

proc plm restore = curlingbyrd;
 lsmeans treatment * dpi / ilink cl adjust = tukey lines;
 lsmestimate treatment*dpi
'SL mite on SL - 14 vs 28d' [1, 1 1] [-1, 1 2],
...
/ adjust = simulate exp ilink cl;
ods output LSMeans = lsmeansout;
run;
```