Engineering the wheat genome to reduce the susceptibility to fungal and viral diseases

by

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B.S., Universidad del Valle, 2004

AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Department of Plant Pathology
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Abstract

Wheat is a major staple crop, providing calories and proteins to millions of people worldwide. Nevertheless, wheat production is constantly threatened by biotic factors such as pests and diseases, causing about 21% annual yield losses. Undoubtably the use of resistant materials is one of the best strategies to manage diseases, but sources of genetic resistance are limited for some diseases. Genetic engineering is a valuable alternative to incorporate resistance to those diseases for which other management strategies have not been effective. In this research, wheat plants were genetically modified with the aim of reducing susceptibility to three major pathogens *Fusarium graminearum* (Fusarium head blight - FHB)*, Magnaporthe oryzae Triticum* pathotype (MoT) (wheat blast - WB), and *Wheat streak mosaic virus* (WSMV). Embryogenic calli of the susceptible cultivar 'Bobwhite' were co-transformed via biolistic with DNA plasmids with the purpose of expressing exogenous genes or editing host genes by CRISPR/Cas9. One of the strategies used to enhance resistance to FHB, wheat spike blast (W_SB) and wheat leaf blast (W_LB), was to expand the basal defense of wheat by expressing genes encoding antimicrobial peptides (AMPs). Twenty transgenic lines independently transformed with four AMP genes (*Ace-AMP1* from onion, *WD* from wasabi, *ARACIN1* from *Arabidopsis*, and *Zeamatin* from maize) were challenged with *F. graminearum*, and four lines, Ace1_8866.A, Wj1_8556.A.4.1, Wj1_8582.A.3, and ARC1_8894.D.1, showed a slight reduction in the percentage of spikelets affected (PSA). Nevertheless the expression of these AMPs did not confer resistance to FHB because the PSAs ranged between 68 and 86%. Significant reductions in the WsB severity (PSA) or W_LB severity (% leaf area affected - PLA) were not observed in any of the sixteen lines evaluated. Another approach used in this research to reduce susceptibility to WB was based on the resistance mediated by the host resistance (*R*) gene – pathogen avirulence (*AVR*) gene

interaction. After assessing the presence of 22 effector genes in 102 South-American MoT isolates, four *AVR* genes *AVR-Piz-t*, *AVR-Pi9*, *AVR-Pi54* and *ACE1* were found in high frequency. The rice *R* gene *Piz-t* was used to transform wheat, and transgenic lines were challenged with MoT isolate T-25. Significant reductions in susceptibility to W_SB were not detected, but the lines Piz-t_5238.C.1 and Piz-t_5503.C1 showed a significant decrease in PLA, suggesting that *Piz-t* could confer some resistance to W_LB. To incorporate resistance to WSMV, the wheat endogenous genes *eIF(iso)4E-2* and *eIF4G*, encoding translation initiation factors which could favor the multiplication of the virus in the host, were CRISPR/Cas9-edited. Four T_0 plants with mutations in the target site were recovered. T_2 plants from edited lines 4385 (six *eIF(iso)4E-2* alleles mutated) and 5697 (*eIF4G* alleles mutated in the A and/or D genome) were challenged with WSMV isolate 'Sidney 81'. Expression levels of the targeted genes in edited-lines were reduced, compared to control Bobwhite_wild-type. However, characteristic WSMV symptoms developed both in edited-lines and in Bobwhite_wild-type, and differences in virus accumulation were not found, suggesting that the knockout of these genes had no effect on virus infection. Implementation of new CRISPR-based genome editing technologies should be considered to introduce resistance to these diseases in wheat.

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Approved by:

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Dedication

To those who accompanied me on this journey, even in the distance, and who have believed in me, more than myself.

Chapter 1 - Introduction

Bread wheat (*Triticum aestivum*) is a major staple crop, cultivated and consumed worldwide. Wheat is the most planted cereal, the second in production after maize, and it provides almost 25% of calories and 20% of the protein daily intakes (http://www.fao.org/faostat/en/#data). In 2018, 735 million tonnes were harvested worldwide, and the United States ranked as the fifth producer, with 51.3 million tonnes (1.89 billion bushels) (USDA, 2019). In 2019, wheat production in the U.S. was worth US$8.88 billion, and Kansas was the major producer, with 338 million bushels valued at US$1.37 billion (https://www.nass.usda.gov/Data_Visualization/Commodity/index.php).

Although yields of wheat and other main crops increase year by year, the pace is not enough to meet the food demand of the growing population. According to FAO (2017), global food production must increase more than 50% to meet these needs. The limited arable land and climate change, that brings droughts, floods and extreme temperatures, pose an additional challenge to agriculture. Biotic factors such as pests and diseases have an additional negative impact on crop production. According to Savary et al. (2019), the estimated global losses caused by pests and diseases in five of the major food crops (wheat, rice, maize, potato and soybean) range between 17 and 30%, with an average loss of 21.5% for wheat. An efficient and sustainable management of pests and diseases is fundamental for food security.

About one hundred diseases affecting wheat have been reported, although not all of them have an economic impact on wheat production, and several of them are restricted to specific producing regions (Bockus et al., 2010). In Kansas, fifteen diseases, including lesion nematodes, are the most prevalent and they caused a cumulative estimated yield loss of 14.6% in 2019, equivalent to 51.2 million bushels (Hollandbeck et al., 2019). Worldwide, fungal diseases cause

the most significant impact on wheat production, followed by viral diseases (Serfling et al., 2016). Nematodes and bacteria can also cause severe damages.

Among fungal diseases, rusts diseases are among the most limiting and widely distributed. There are three wheat rust diseases, stripe or yellow rust caused by *Puccinia striiformis* f. sp. *tritici* (*Pst*), leaf or brown rust caused by *P. triiticina* (*Pt*), and stem or black rust caused by *P. graminis* f. sp. *tritici* (*Pgt*) (Singh et al., 2016). Stripe rust is considered the most economically important wheat rust disease, causing severe losses and affecting most commercial varieties (Figueroa et al., 2018). *Pst* populations adapt quickly, and the breakdown of several resistance genes, the expansion to regions with higher temperatures and the emergence of new and more aggressive race groups have been documented (Ali et al., 2014; Hovmøller et al., 2015; Kolmer, 2005). Leaf rust is the most common and widely distributed (Kolmer, 2005). The disease causes a significant reduction in grain weight and number of grains per head (Figueroa et al., 2018). *Pt* is highly diverse, adapts to several climates and its virulence increases in response to the leaf rust-resistant cultivars quickly (Serfling et al., 2016). Stem rust is very aggressive, widely distributed, and it has caused epidemics in major wheat producing areas (Figueroa et al., 2018). Stem rust has been controlled in several growing regions due to the use of resistant materials and removal of the alternate host (barberry), nevertheless the emergence of more virulent populations which overcome genetic resistance, like the Ug99 race and related races, are threatening production in several growing regions to which they have expanded (Singh et al., 2015; Singh et al., 2016). Leaf spotting diseases include tan spot and Septoria complex or Septoria blotch. Tan spot (TS), caused by *Pyrenophora tritici-repentis* (Ptr), is a significant disease occurring in all wheat growing areas (Faris et al., 2013). The fungus causes severe tissue damage, with chlorotic and necrotic symptoms due to the production of toxins, reducing the

photosynthetic area and reducing the number of grains per head and grain weight (Figueroa et al., 2018; Serfling et al. 2016). Septoria complex includes the diseases Septoria tritici blotch, caused by *Zymoseptoria tritici* (formerly *Mycosphaerella graminicola* or *Septoria tritici*), which is the main leaf disease in temperate growing regions, Septoria leaf blotch, caused by *S. avenae* f. sp. *triticea* (teleomorph *Leptosphaeria avenae* f. sp. *triticea*), and Septoria nodorum blotch, caused by *Parastagonospora nodorum*, which can infect glumes and leaves (Figueroa et al., 2018; Serfling et al., 2016; Singh et al., 2016). Infection with Septoria results in reduced seed set, low seed filling and shriveled grains (Serfling et al., 2016). Two important diseases cause damage in wheat heads, Fusarium head blight (FHB) and wheat blast (WB). FHB is caused by several species of *Fusarium*, but *Fusarium graminearum* is the most important globally (Singh et al., 2016). FHB is widely distributed in wheat growing regions, and epidemics occur every four or five years (Figueroa et al., 2018). Humid and warm environments during anthesis favor the disease, and the most characteristic symptom of FHB in the field is the bleaching of individual spikelets or the entire head (McMullen et al., 2012). The infection of heads during anthesis results in poor grain development with low weight, shriveled and discolored kernels (McMullen et al., 2012). In addition, grains are contaminated with sesquiterpenoid trichothecene mycotoxins, which is a health risk for humans and animals (Figueroa et al., 2018). Wheat blast disease, caused by *Magnaporthe oryzae Triticum* pathotype, is a recently emerged disease that was initially reported in Brazil in 1985 (Cruz & Valent, 2017). WB spread to other wheat producing countries in South America and it was restricted to that region until it was reported in Bangladesh in 2016 (Ceresini et al., 2019; Malaker et al., 2016). Infection of the rachis by the pathogen results in bleaching of the head, affecting the grain development and reducing the yield and grain quality (Duveiller et al., 2016).

Viral diseases cause important losses in wheat production worldwide, some of the most economically damaging diseases are the barley yellow dwarf (BYD) complex, the wheat streak mosaic (WSM) complex and the soilborne viruses (Rotenberg et al., 2016). BYD complex is the most economically important viral disease of cereals globally, affecting wheat, barley, rice, maize, oat and rye-grass (Miller & Rasochova, 1997)). The disease is caused by the infection of one or more virus species in the family *Luteoviridae*, genera *Luteovirus* (*Barley yellow dwarf virus* – BYDV spp. PAV, PAS, MAV and GAV) or *Polerovirus* (*Cereal yellow dwarf virus* – CYDV-RPV, CYDV-RPS; Maize yellow dwarf virus-RMV), or not assigned to a genus (BYDV-SGV, BYDV-RMV and BYDV-GPV) (Miller & Rasochova, 1997). All these viruses are phloem-restricted and transmitted in a persistent manner by different species of aphids (Halbert & Voegtlin, 1995). Damage of the phloem by BYD viruses cause dwarfing, chlorosis, stunting, reduction in number of florets per head, ears per plant and grain weight (Choudhury et al., 2017). The WSM complex disease is caused by the infection of up to three viruses, *Wheat streak mosaic virus* (WSMV, family *Potyviridae*, genus *Tritimovirus*), *Triticum mosaic virus* (TriMV, family *Potyviridae,* genus *Poacevirus*), and *High Plains wheat mosaic virus* (HPWMoV, family *Fimoviridae*, genus *Emaravirus*), all of them vectored by the wheat curl mite (WCM, *Aceria tosichella* Keifer) (Rotenberg et al., 2016). TriMV and HPWMoV are mainly distributed in the Great Plains in the U.S., (Byamukama et al., 2013; Stewart et al., 2013) although HPWMoV has been reported in Argentina and Australia (Alemandri et al., 2017; Coutts, et al. 2014); but WSMV is distributed in most wheat-growing regions, infecting several species of the family *Poaceae* (Singh et al. 2018). Upon virus infection, the photosynthetic capacity, root biomass and water use efficiency are reduced, causing low tillering, low number of spikes, and kernels poorly filled with reduced weight (Hadi et al., 2011; Price et al. 2010; Singh et al., 2018). In co-

infections, the synergism between WSMV and TriMV causes more severe symptoms (Tatineni et al., 2010). The soilborne viruses most important to Kansas and widely distributed in the U.S. are the *Soilborne wheat mosaic virus* (SBWM, family *Virgaviridae*, genus *Furovirus*) and *Wheat spindle streak mosaic virus* (WSSMV, family *Potyviridae*, genus *Bymovirus*) (Rotenberg et al., 2016). Both viruses are transmitted by the parasite plasmodiophorid *Polymiyxa graminis*, which has a worldwide distribution, multiplies in the roots of several cereals, and can persist in soil for several years (Kühne, 2009). These viruses diminish the plant vigor, reducing the tillering, and the number of grain per spike and per plant (Kühne, 2009).

Several of these fungal and viral diseases can be managed efficiently by cultural practices, chemical control or planting of resistant cultivars. Rusts can be controlled by application of some fungicides, and cultural practices like the eradication of alternative host and the removal of inter-crop 'green bridges' can also help to mitigate the diseases (Figueroa et al., 2018). More than 187 rust resistance genes have been described in common wheat, durum and wild relatives (Aktar-Uz-Zaman et al., 2017), and several commercial varieties with resistance to rusts have been developed, although the breakdown of resistance by the emergence of new and more aggressive races is a challenge for the deployment of durable resistance (Kolmer, 2005). Tan spot and Septoria diseases are managed by chemical control, cultural practices, and host resistance, and so far, the genetic resistance deployed has been durable (Figueroa et al., 2018; Singh et al., 2016). BYD can be partially controlled by the use of insecticides and some cultural practices, like changes in planting date, crop rotation, and removal of virus reservoirs, but the use of materials with resistance or tolerance is the most effective control method, and although sources of resistance to BYD have not been found in the primary gene pool, some resistance has been introduced from *Thinopyrum intermedium* in wheat (Choudhury et al., 2017; Serfling et al.

2016). Fungicides and cultural practices have not shown any effect on soilborne virus diseases, but several commercial cultivars with resistance are available, and they have shown durable resistance (Bockus et al., 2001; Kühne, 2009).

The managing of diseases such as FHB, WB and WSM has been a challenge, either because chemical control or cultural practices have no significant effect on control, or because few sources of genetic resistance have been identified, or because the resistance displayed has not been sufficient to manage the disease. Although several sources of resistance to FHB have been identified, the introduction of resistance in elite materials has been difficult because the resistance is quantitative and conditioned by the environment (Steiner et al., 2017). Fungicides can provide some control of FHB, but the application timing is crucial for an effective control (McMullen et al., 2012). Although the use of fungicides can be effective for the management of WB under disease conducive conditions, fungicide efficacy depends on region and disease pressure (Cruz et al., 2019). Five genes conferring resistance to MoT isolates have been identified (Anh et al., 2015; Tagle et al., 2015; Wang et al., 2018; Zhan et al. 2008), but some of them are temperature sensitive, and/or their effectiveness is low against more recent MoT isolates (Cruz & Valent, 2017). Wheat materials carrying the 2NS/AS chromosomal translocation from the wild wheat *Aegilops ventricosa* have shown resistance to MoT (Cruz et al., 2016), and this has been the most significant source of resistance so far. However, not all materials with the 2NS translocation perform well against MoT, which seems to be associated with the genetic background or environmental effects (Cruz et al., 2016), and new and apparently more aggressive MoT isolates are breaking the resistance conferred by the 2NS translocation (Cruppe et al., 2020). Regarding WSM disease complex control, the best strategy has been the elimination of "green bridges" like voluntary wheat, maize and other grasses, which remain

green during the summer, serving as reservoirs of the vector and viruses (Tatineni & Hein, 2018). Three resistance genes have been identified (Friebe et al., 1991; Haley et al., 2002; Liu et al., 2011) and transferred to wheat cultivars. *Wsm2* confers resistance to *WSMV,* whereas *Wsm1* and *Wsm3* are effective against *WSMV* and *TriMV*. The three genes are temperature sensitive, *Wsm1* and *Wsm2* are effective at or below 18°C, while *Wsm3* is still effective against *WSMV* at 24°C. (Haley et al., 2011; Kumssa et al., 2017; Seifers et al., 1995; Seifers et al. 2007; Tatineni et al., 2010). The emergence of more virulent strains and the increase in temperature pose a risk to the durability of resistance associated with these genes.

Conventional breeding has been successful incorporating disease resistance in many cases. However, when sources of genetic resistance are scarce, or populations of the pathogen are changing, genetic engineering is a promising solution, not only to incorporate resistance directly, but also to expand the genetic diversity. According to Dong and Ronald (2019), genetic engineering also has some advantages compared to conventional breeding, for example, the incorporation of the desired trait is achieved in less time and without dragging along unwanted traits. Also, there is a high availability of genes because the exchange is not restricted by the species; and the introduction of a trait in vegetatively propagated crops is more efficient. There are two approaches to modify the genome, (i) conventional transgenesis, where a coding sequence is introduced into the host genome to express/overexpress a desired protein or repress the expression of another gene, such as gene silencing mediated by RNA interference; and (ii) genome editing, where specific gene changes are introduced by nucleases that are specifically guided to the target gene, including the zinc-finger nucleases - ZFNs (Urnov et al., 2010), transcription activator-like effector nucleases - TALENs (Miller et al., 2010) and clustered regularly interspaced short palindromic repeats - CRISPR (Doudna & Charpentier, 2014).

Over-expression of wheat endogenous genes under the control of constitutive promoters, such as the maize ubiquitin promoter, or the expression of exogenous genes have been used to confer resistance to several wheat diseases. For example, wheat transgenic plants overexpressing some alleles of the powdery mildew resistance locus *Pm3*, were significantly more resistant to powdery mildew (caused by *Blumeria graminis* f. sp. *tritici*) than the non-transgenic plants in field experiments (Brunner et al., 2011). Likewise, reductions in FHB severity, DON accumulation, and in the percentage of visually affected kernels were observed in greenhouse and field experiments using wheat transgenic lines overexpressing the wheat α-1-purothionin, barley thaumatin-like protein 1(tlp-1), and barley β-1,3-glucanase (Mackintosh et al., 2007). The *Arabidopsis thaliana NPR1* gene (regulator of the systemic acquired resistance -SAR) expressed in transgenic wheat induced a fast and high accumulation of the endogenous pathogenesis-related 1 (PR1) transcripts upon *F. graminearum* inoculation, and pathogen was mainly restricted to the inoculated spikelet (Makandar et al., 2006). Expression of a barley UDP-glucosyltransferase (HvUGT13248) in wheat resulted in significantly higher resistance to disease spread in the spike, likely due to an increased ability to metabolize DON to a less-toxic derivate (Li et al., 2015). The gene *pac1* from *Schizosaccharomyces pombe*, which encodes a double-stranded RNA-specific RNase III, was transformed into wheat, and several levels of resistance to the BYDV-GPV infection were observed, from plants severely affected to plants without symptoms (Yan et al., 2006). Genes encoding antimicrobial peptides (AMPs), which are small peptides (usually less than 100 amino acids) that are part of the innate immune system of almost all organisms (Campos et al., 2018) have also been used to enhance disease resistance in wheat. Expression of the synthetic peptides MsrA2 and 10R resulted in significant reductions in FHB susceptibility (Badea et al., 2013), whereas the expression of RsAFP2, a plant defensin from seeds of radish,

conferred enhanced resistance to FHB and sharp eyespot (caused by *Rhizoctonia cerealis*) (Li et al., 2011). The over-expression of the wheat lipid transfer protein gene *TaLTP5* resulted in increased resistance to FHB and common root rot, caused by *Cochliobolus sativus* (Zhu et al., 2012).

The RNA interference (RNAi) mechanism has been exploited to silence pathogen or host-endogenous genes by the expression of antisense or hairpin RNAi constructs (Boriskuj et al., 2019). For example, hairpin RNA-enconding constructs (hpRNA) targeting the replicase gene of BYDV-GPV and the polymerase gene of BYDV-PAV were successfully used to induce resistance to the respective strains (Yan et al., 2007; Yassaie et al., 2011). Likewise, full-length gene constructs or hpRNA constructs based on the genes *NIb* (Nuclear Inclusion polymerase), *CP* (coat protein), and *NIa* (Nuclear Inclusion proteasa) of WSMV were used to induce resistance in wheat transgenic plants (Cruz et al., 2014; Fahim et al., 2010; Li et al., 2005; Sivamani et al., 2000; Sivamani et al., 2002). Tatineni et al. (2020) used a hpRNA construct with fragments of the *NIb* genes from WSMV and TriMV to transform wheat, and plants with resistance to one or both viruses were recovered. The *F. graminearum chitin synthase* gene (*Ch3b*) was silenced by the expression of hpRNA constructs in wheat, and transgenic lines showed high levels of stable and durable resistance to FHB (Cheng et al., 2015). To enhance resistance to several viruses, Rupp et al. (2019) used RNAi to silence the wheat endogenous genes *eIF(iso)4E-2* and *eIF4G*, which are part of the eIF4 and eIF(iso)4 transcription initiation complexes, respectively. The authors reported that *eIF(iso)4E-2*- and *eIF4G*- silenced lines were resistant to WSMV, TriMV and SbWMV (Rupp et al., 2019).

Gene editing technologies have been used mainly to knock-out susceptibility genes. The first successful example of using gene editing to modify multiple alleles (homeoalleles) in wheat

was the edition of the susceptibility gene *MLO* (Mildew-Resistance Locus) using TALEN (Wang et al., 2014). Authors reported that plants with mutations in the six homeoalleles were highly resistant to *B. graminis* f. sp. *tritici*, and they also described the use of CRISPR/Cas9 to introduce mutations in *MLO* alleles in the A genome (Wang et al., 2014). The knockdown (mediated by RNAi) and knockout (CRISPR/Cas9 mediated) of the *enhanced disease resistance 1* (*EDR1*) gene, encoding a Raf-like mitogen-activated protein kinase kinase kinase, improved the resistance to powdery mildew in silenced and edited plants (Zhang et al., 2017). The resistance to FHB was significantly enhanced by the knockdown and knockout of the wheat gene *TaHRC*, which encodes a putative histidine-rich calcium-binding protein (Su et al., 2019). Likewise, the CRISPR/Cas9 edition of the endogenous *TaNFXL1* gene, which is a DON-induced transcription factor, improved resistance to FHB (Brauer et al., 2020).

The challenge of increasing food production on the same available arable land forces us to reduce losses caused by diseases. It is then a necessity to implement durable, sustainable, economic, and environmental-friendly strategies to manage diseases. The potential of genome modification, and particularly genome editing, to introduce disease resistance has brought a lot of attention to this area. In line with this purpose, this study aimed to evaluate the effectiveness of some biotechnological approaches to reduce the susceptibility of wheat to three diseases: Fusarium head blight (FHB), Wheat blast (WB), and *Wheat streak mosaic virus* (WSMV). The objectives associated with this general goal were:

- Identify and select antimicrobial peptides (AMPs) with potential activity against *F. graminearum* and *M. oryzae Triticum* pathotype (MoT).
- Develop wheat transgenic lines expressing AMPs and evaluate the response of these lines to FHB and WB.

- Characterize the presence of genes homologous to the *M. oryzae Oryza* pathotype-effector genes in a group of South American MoT isolates, and identify cognate rice resistance genes with the potential to confer resistance to MoT.
- Develop wheat transgenic lines expressing the rice resistance gene, and evaluate its potential to enhance the resistance to MoT.
- Generate wheat lines with the endogenous genes *eIF(iso)4E-2* and *eIF4G* edited by CRISPR/Cas9, and test the resistance of these lines to WSMV.

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Chapter 2 - Use of antimicrobial peptides (AMPs) to reduce the susceptibility of wheat to Fusarium head blight (FHB)

Abstract

Fusarium head blight (FHB) is a devasting disease affecting cereals like wheat (*Triticum aestivum* L.) and barley (*Hordeum vulgare* L.). FHB is caused by several *Fusarium* species, *F. graminearum* being the most prevalent. The disease has a strong impact, not only because it reduces the yield and quality of the seed, but because the pathogen produces mycotoxins that contaminate the grain. FHB is distributed worldwide and devasting epidemics affecting wheat have occurred regularly, especially in China and the USA. Genetic sources of resistance to FHB have been identified in hexaploid, tetraploid, and diploid wheat, as well as in close wheat relatives, but this resistance is a quantitative trait, which has hindered the development and release of resistant materials. Biotechnological tools, such as the genetic transformation of wheat, are valuable approaches that can help improve resistance to FHB. Plant Antimicrobial Peptides (AMPs) have shown a wide range of action against different plant pathogens. The plant peptides Ace-AMP1, WD, ARACIN1, and Zeamatin, from onion (*Allium cepa*), wasabi (*Eutrema japonicum*), *Arabidopsis thaliana*, and maize (*Zea mays*), respectively, have shown antifungal activity previously. The aim of this study was to evaluate the potential of these four peptides to reduce susceptibility to FHB. Genes encoding the four plant peptides mentioned were independently used to transform embryogenic calli of the wheat susceptible cultivar 'Bobwhite' via biolistics. Thirty-two transgenic plants (T_0) carrying and expressing the AMP genes were recovered. Plants of 20 transgenic lines were challenged with *F. graminearum*. The percentage of spikelets with FHB symptoms was scored at several points (days) after inoculation. The Area

Under the Disease Progress Curve (AUDPC) was calculated and used to evaluate the response of the lines to the disease. Plants from four transgenic lines, Ace1_8866.A (T_1), Wj1_8556.A.4.1 (T_3), Wj1_8582.A.3 (T_2), and ARC1_8894.D.1 (T_2 and T_3), showed a reduction in the percentage of symptomatic spikelets and in the AUDPC. Although the AUDPC of the four transgenic lines mentioned were statistically different compared to other transgenic lines and controls, the percentages of spikelets affected at the end of the evaluation (14 or 16 days after inoculation) ranged between 68 and 86 %. Taken together, the results showed that the peptides Ace-AMP1, WD, ARACIN1, and Zeamatin did not confer resistance to FHB, and therefore, more efforts should be made to identify genes with high potential, which may contribute to effective control of this disease.

Introduction

Fusarium head blight (FHB), also known as scab, is one of the most destructive diseases of cereals like wheat and barley. In the USA, from 1993 to 2014, losses in hard, soft and durum wheat due to FHB valued $17 billion in total (Wilson et al., 2017). In Kansas, this disease was ranked as the third most important disease in 2019, causing a yield loss of 2.1% or 7.6 million bushels (Hollandbeck et al., 2019). Several *Fusarium* species are associated with FHB, but *Fusarium graminearum* Schwabe (teleomorph *Gibberella zeae* (Schwein.)), is the prevalent species in North America and other regions (Gale 2003; Shaner 2003). Wheat is susceptible to infection from anthesis (10.51 Feekes scale) through the soft dough stage of kernel development (11.2 Feekes scale), and the infection results in whitening or bleaching of the entire head or portions thereof (McMullen et al., 2012). The disease results in direct yield and quality reduction due to poor grain development or because grains are shriveled, discolored, and with reduced weight (McMullen et al., 2012). Indirect losses are the result of grain contamination with

trichothecene mycotoxins produced by the *Fusarium* species, particularly deoxynivalenol (DON), making them unsuitable for human consumption and as livestock feed (Ma et al., 2020).

The pathogen over-winters on infested crop residues of several plant species as chlamydospores or mycelia, making crop residues the main reservoir of inoculum (Wegulo et al., 2008; Parry et al., 1995). When conditions are favorable (warm, humid and wet), perithecia form on the surface of residues, and ascospores are discharged into the air (Schmale III & Bergstrom, 2003). Asexual spores (macroconida) are also produced on infested residues, and they are dispersed by rain splashing or wind (Schmale III & Bergstrom, 2003). Ascospores and macroconidia landing on wheat heads initiate infection (Wegulo et al., 2008). When the onset of wheat anthesis coincides with warm and moist weather conditions, epidemics occur (Parry et al., 1995); the risk of epidemics increases when the relative humidity is above 70% (Dweba et al., 2017).

FHB control strategies include cultural practices, irrigation management, chemical control, biological control, disease forecasting, use of resistant or tolerant cultivars, and harvesting strategies; the use of two or more strategies is highly recommended (Wegulo et al., 2015). The use of resistant cultivars is the most effective control strategy, but because the FHB resistance is inherited quantitatively and influenced by environmental factors, the development of resistant materials by breeding has been slow (Wegulo et al., 2015; Steiner et al., 2017). Several sources of genetic resistance have been reported in hexaploid wheat germplasm (Steiner et al., 2017), but only a few of these germplasm sources have shown high levels of resistance, and they do not exhibit immunity (Bai et al., 2018). FHB resistance has also been discovered in wheat subspecies and its close relatives, including diploid and tetraploid wheat species (Buerstmayr et al., 2019; Ma et al., 2020). More than 100 quantitative trait loci (QTLs) have

been identified for resistance to FHB, although the list is reduced to 50 QTLs when unique chromosome locations are considered, and only seven of these have been formally assigned a gene name (*Fhb*1 to *Fhb*7) (Bai et al., 2018). Based on host response to pathogen infection, five types of resistance to FHB and DON have been described: resistance to initial fungal infection (type I), resistance to spread of infection within a spike (type II), resistance to accumulation of toxins or ability to degrade them (type III), resistance to kernel infection (type IV), and infection without substantial effect on yield and quality or tolerance (type V) (Shroeder & Christensen, 1963; Miller et al., 1985; Mesterházy, 1995; Mesterházy et al., 1999).

Due to complexity in the introgression of resistance by conventional breeding, several genetic engineering approaches have been explored to try to improve resistance to FHB. These approaches include the expression or overexpression of endogenous genes related to the basal defense response or with the defense signaling, or the introduction of genes from alien species like genes related to detoxification or encoding antimicrobial peptides (Ma et al. 2020). For example, the overexpression of wheat α-1-purothionin, barley thaumatin-like protein 1(tlp-1), and barley β-1,3-glucanase in transgenic wheat lines resulted in reductions in FHB severity of more than 30% in greenhouse evaluations and significant reductions in percent FHB severity, DON concentration, and percent of visually scabby kernels in field evaluations (Mackintosh et al., 2007). Wheat transgenic plants expressing the *Arabidopsis thaliana NPR1* gene (regulator of the systemic acquired resistance -SAR) showed a faster and higher accumulation of endogenous pathogenesis-related 1 (PR1) transcripts than non-transgenic plants when they were challenged against *F. graminearum*. This enhanced response resulted in a type II resistance, where the pathogen was mainly restricted to the inoculated spikelet (Makandar et al., 2006). Transgenic wheat events expressing a barley UDP-glucosyltransferase (HvUGT13248) had significantly

higher resistance to disease spread in the spike, likely due to an increased ability to conjugate DON with glucose to the less-toxic DON-3-O-glucoside (D3G) (Li et al., 2015). Incorporation of Antimicrobial Peptides (AMPs), like the synthetic peptides MsrA2 (modified dermaseptin B1 from the frog *Phyllomedusa bicolor*) and 10R (variant of the indolicidin, present in cytoplasmatic granules of bovine neutrophils), under the control of several tissue-specific promoters in wheat transgenic plants (cv. Fielder) resulted in a reduction of 50% in FHB susceptibility (Badea et al., 2013). Likewise, the constitutive expression of RsAFP2, a plant defensin from seeds of radish (*Raphanus sativus*), in transgenic wheat conferred enhanced resistance to FHB and sharp eyespot (caused by *Rhizoctonia cerealis*) (Li et al., 2011). Alternatively, the silencing of *Fusarium* or host genes mediated by RNA interference (RNAi) has showed promising results. Cheng et al. (2015) reported that two wheat transgenic lines expressing RNAi constructs targeting the chitin synthase 3b (Chs3b) of *F. graminearum* showed a significant reduction in the percentage of infected spikelets through multiple generations, under controlled conditions and under natural infection in the field. In a recent study, the knockdown (mediated by RNAi) and knockout (CRISPR/Cas9 mediated) of the wheat gene *TaHRC*, which encodes a putative histidine-rich calcium-binding protein and likely is a susceptibility gene, significantly enhanced the resistance to FHB (Su et al., 2019). Brauer et al. (2020) used CRISPR/Cas9 to edit the wheat endogenous *TaNFXL1* gene, which is a DON-induced transcription factor, and the *TaNFXL1*-edited plants showed a strong reduction in the gene expression (60 to 99%) and an improved resistance to FHB.

Antimicrobial peptides (AMPs), also known as host defense peptides, are small and generally positively charged peptides with the ability to eliminate microbial pathogens and produced by virtually all organisms (Mahlapuu et al., 2016). Plant AMPs are small cationic

peptides (2 to 10 kDa), amphipathic, rich in cysteine, usually stabilized by 2 to 6 disulfide bridges, with high thermostability (Stotz et al., 2013). Most plant AMPs are processed from a precursor with three domains: an N-terminal signal peptide, the mature peptide, and a C-terminal pro-domain (Tam et al., 2015). In plants, AMPs can act directly against targets, including fungi, oomycetes, bacteria, nematodes and some herbivorous insects, mainly by damaging their cell membranes, but they can also act indirectly modulating defense responses and defense pathways mediated by mitogen-activated protein kinases (MAPKs), reactive oxygen species, hormones and sugar signaling (Bolouri Moghaddam et al., 2016; Campos et al., 2018). Several types of plant AMPs have been described, and most of them have been classified into cyclotides, defensins, lipid transfer proteins (LTPs), thionins, snakins, hevein-like peptides, knottin-type peptides, and others (Goyal & Mattoo, 2016). The proven activity against various phytopathogenic fungi makes plant AMPs potential candidates for improving wheat resistance to *F. graminearum*. Among the vast diversity of plant AMPs, four were selected for this study: Ace-AMP1, WD, ARACIN1 and Zeamatin.

Ace-AMP1 isolated from onion (*Allium cepa*) seeds is a lipid transfer protein with a 76% homology and structural analogies to plant non-specific lipid transfer proteins (ns-LTPs) (Cammue et al., 1995). ns-LTPs are small and basic proteins, whose activity *in vitro* is associated with binding and transfer of lipids between membranes, but whose *in vivo* activity has not been elucidated, although it has been suggested to have an extracellular role (Liu et al., 2015). *In vitro* assays using Ace-AMP1 purified from onion showed strong antifungal activity, inhibiting the growth of different fungi (*Alternaria brassicola, Ascochyta pisi, Botrytis cinerea, Colletotrichum lindemuthianum, Fusarium culmorum, Fusarium oxysporum* f.sp. *pisi, F. oxysporum* f.sp. *lycopersici, Nectria kaematococca, Phoma betae, Pyrenophora tritici-repentis, Pyricularia*

oryzae and Verticillium dahlia) with 50% growth inhibitory concentration (IC_{50}) values ranging from 0.25 to 10 μg mL^{-1} (Cammue et al., 1995). In addition, Ace-AMP1 was also active against Gram-positive bacteria with IC_{50} values of 0.8 to 8 μg mL^{-1} (Cammue et al., 1995). According to Wu et al. (2011), Trx-Ace-AMP1, a fusion protein expressed in *Escherichia coli*, inhibited the growth of the plant pathogenic fungi *Alternaria solani* (IC_{50} = 20 μg mL^{-1}), *F. oxysporum* f. sp. *vasinfectum* (IC_{50} = 25 μg mL^{-1}), and *Verticillium dahlia* (IC_{50} = 60 μg mL^{-1}). In addition, the external application of the protein to tomato plants improved the resistance to *A. solani*, suggesting that it could be used as a bio-fungicide. *In planta* activity of Ace-AMP1 has been evaluated in several plant species. Transgenic rose lines expressing *Ace-AMP1* showed enhanced resistance to powdery mildew (*Sphaerotheca pannosa*) in detached-leaf and whole-plant assays (Li et al., 2003). Likewise, rice transgenic lines challenged with *Magnaporthe grisea*, *Rhizoctonia solani* and *Xanthomonas oryzae*, showed reductions of 86%, 67% and 82% in disease severity, respectively (Patkar & Chattoo, 2006). Transgenic wheat lines expressing *Ace*-AMP1 showed a 20% to 50% increase in resistance to *Blumeria graminis* f. sp. *tritici*. In addition, increased levels of salicylic acid and over expression of defense-related genes were observed when ears were inoculated with *Neovossia indica* (Roy-Barman et al., 2006). Roots of bananas (Rasthali, AAB, Silk gp) expressing *Ace*-AMP1 challenged with *F. oxysporum* f. sp *cubense* race 1 (Foc) showed low root colonization and less symptoms compared to control plants (Mohandas et al., 2013).

WD is a small plant defensin encoded by the gene *WT1* (or *WD* gene), isolated from Wasabi plants (*Eutrema japonicum*) by Saitoh et al. (2001). Plant defensins, previously known as gamma-thionins, are small (45 to 54 amino acids) cysteine-rich and highly basic peptides, widely distributed among dicots and monocots (Stotz et al., 2013). They have shown higher

effectiveness against fungi than bacteria. Although its mode of action is unclear, it has been suggested that it can produce disruptions and destabilization of membranes, resulting in inhibition of fungal growth due to morphological distortions of hyphae (Nawrot et al., 2014). WD purified from potato virus X (PVX)-infected tobacco leaves showed *in vitro* antifungal activity against *M. grisea* and *B. cinerea,* with IC_{50} values of 5 μg mL^{-1} and 20 μg mL^{-1}, respectively (Saitoh et al., 2001). Later, the same group reported that transgenic rice lines overexpressing *WT1* showed various levels of susceptibility to *M. grisea*, with some lines as resistant as the resistant cultivar used as the control, and other lines as susceptible as the susceptible control (Kanzaki et al., 2002). A reduction in symptoms was also observed in detached leaves and in whole seedlings of *WD* transgenic lines of 'Egusi' melon (*Colocynthis citrullus* L.) when they were challenged with *F. oxysporum* and *A. solani* (Ntui et al., 2010). Kong et al. (2014) reported that plantlets of transgenic tobacco and tomato lines expressing *WD* showed resistance to growth and proliferation of *F. oxysporum*, and less symptoms compared to the non-transgenic plants. The authors also found that crude protein extracts obtained from roots and leaves of transgenic tobacco and tomato lines inhibited the *in vitro* growth of *F. oxysporum* (Kong et al., 2014). According to Khan et al. (2006), the expression of *WD* in transgenic potato plants partially restricted growth of *Botrytis cinerea*. Potato transgenic lines co-expressing the *ChiC* (chitinase gene from *Streptomyces griseus*) and *WD* genes were more resistant to *F. oxysporum* and *A. solani*, than non-transformed plants and lines expressing only one of the transgenes (Khan et al., 2014).

ARACIN1 is a small, cationic, hydrophobic and secreted peptide identified in *Arabidopsis*. *ARACIN1* and *ARACIN2* genes are lineage specific to the *Brassicaceae* family and they are transcriptionally regulated by biotic and abiotic stresses (Neukermans et al., 2015).

Chemically synthesized ARACIN1 showed *in vitro* antifungal activity against *Alternaria brassicicola, B. cinerea*, *Fusarium graminearum*, *Sclerotinia sclerotiorum* and *Saccharomyces cerevisiae*, with IC_{50} values of 5.46 μg mL^{-1}, 3.05 μg mL^{-1}, 28.17 μg mL^{-1}, 0.73 μg mL^{-1}, and 6.41 μg mL^{-1}, respectively. Furthermore, transgenic *Arabidopsis* plants overexpressing ARACIN1 exhibited a reduction in disease symptoms when they were inoculated with *A. brassicicola* and *B. cinerea* (Neukermans et al., 2015).

Zeamatin is a small protein (22 kDa) purified from *Zea mays* seeds (Roberts & Selitrennikoff, 1990). The protein has a high homology to thaumatin and to thaumatin-like proteins, which are part of the pathogenesis-related group 5 (PR-5) proteins, and whose expression is associated with the response to environmental stresses, including pathogens, pests, drought, wounding and cold hardiness (Vigers et al., 1991; Liu et al., 2000). The purified protein from maize, from transgenic *Arabidopsis* and tomato plants, showed a potent antifungal activity, inhibiting the growth of *Candida albicans, Neurospora crassa, A. solani* and *Trichoderma reesei* at concentrations as low as 5 μg of zeamatin per ml of minimal culture medium or 10 μg of the purified protein in disk assays in agar (Roberts & Selitrennikoff, 1990; Malehorn et al., 1994). According to Roberts and Selitrennikoff (1990), the protein permeabilizes membranes by direct insertion into the fungal membranes forming pores.

The objectives of this study were to develop transgenic wheat lines independently expressing the genes *Ace-AMP1*, *WD*, *ARACIN1*, and *Zeamatin*, and to evaluate the potential of these peptides to reduce susceptibility to FHB. We hypothesize that these peptides can inhibit or reduce the growth of *F. graminearum in planta* and increase resistance to FHB.

Materials and Methods

AMPs and plasmids

Four peptides were selected for this study. Ace-AMP1 is synthesized as a preprotein of 132 amino acids, with an N-terminal signal peptide (predomain) of 27 amino acids (aa) and a C-terminal prodomain of 12 aa, which are absent from the mature protein (Cammue et al., 1995). WD mature protein is 51 aa and has a 29 aa signal peptide (Saitoh et al., 2001). ARACIN1 is a preprotein of 76 aa, with a predomain and prodomain of 22 and 14 aa, respectively (Neukermans et al., 2015). Zeamatin preprotein is 227 aa, with a predomain of 20 aa (Vigers et al., 1991). The gene sequences were wheat codon optimized by GenScript (Piscataway, NJ), based on the peptide sequences reported in the EMBL nucleotide sequence database (Baker et al., 2000): AF004946 (*Ace-AMP1*), AB012871(*WD*), U06831 (*Zeamatin*), and in the *ARACIN1* sequence reported by Neukermans et al. (2015). The codon-optimized genes were synthesized and cloned into the pAHC17 vector by GenScript (Piscataway, NJ). The genes were cloned under the control of the maize *Ubiquitin1* (*Ubi-1*) promoter and *Nopaline Synthase* terminator (*tNOS*). The plasmids were named as follows: Ace1_pAHC17, Wj1_pAHC17, ARA1_pAHC17, and Zma_pAHC17. The plasmid pAHC20 contains the *bar* gene, which confers resistance to the herbicide glufosinate, under the control of the *Ubi1* promoter (Christensen & Quail, 1996). Resistance to the herbicide glufosinate is used for the selection of transgenic plant tissue during the tissue culture and to identify putative transgenic plants.

Biolistic transformation of wheat

Plants of the spring wheat cultivar Bobwhite (BW; CM33203; released in 1984 by CIMMYT) were used for all transformation experiments. The transformation of wheat embryogenic calli was done according to the protocol described by Tian et al. (2019). Briefly,

wheat spikes were collected 10 to 14 days post-anthesis from Bobwhite plants grown under growth chamber conditions at 20°C/18°C day/night temperature, a 16 h photoperiod, with a light intensity of 450 μE m^{-2} s^{-1}, and 50 to 60% relative humidity. Immature seeds were isolated by hand and surface sterilized with a solution 20% (v/v) bleach and 0.1% (v/v) Tween-20 for 20 minutes under continuous shaking. The seeds were rinsed with sterile double-distilled water (ddH_2O) five times in a laminar flow hood. Immature embryos were excised from the seeds and placed on plates containing the callus induction medium CM4, with the embryo axis faced down in contact with the medium. Plates were incubated for 7 to 10 days in the dark at room temperature (RT, 22 ± 1 °C). Calli regenerated from the scutellum were selected, removed with tweezers and transferred to fresh plates with CM4 medium. Twenty-five calli were placed in the center of each plate, in a 5 x 5 arrangement. Plates were incubated for 2 to 3 days in the dark at RT. On the day of the transformation, the calli were air dried in a laminar flow chamber for 20 min, after removing the petri dish cover. Tungsten particles were washed 3 times with ddH_2O by centrifugation at 10000 rpm for 1 min. Particles were resuspended in 500 μL of ddH_2O and kept on ice. Particles were coated by mixing 25 μL of the particles with 2 μL of any of the AMP plasmids (Ace1_pAHC17, Wj1_pAHC17, ARA1_pAHC17, and Zma_pAHC17) and 2 μL of the plasmid pAHC20 (Christensen & Quail, 1996). Particles and DNA were mixed by vortex and incubated at RT for 1 min. After incubation, 25 μL of 2.5 M $CaCl_2$ and 10 μL of 0.1 M spermidine were added. The solution was mixed by vortex and incubated on ice for 4 min. After the particles settled, 50 μL of the supernatant were removed and discarded. The remaining coated-particle solution was kept on ice, the solution was mixed by vortex before each shot, using 2 μL per shot. Particle bombardment was done using the biolistic system described by Finer et al. (1992), with an helium pressure of 60 PSI. After bombardment, embryogenic calli in

CM4 plates were incubated for 3 to 4 days in the dark at RT. After incubation, the calli were transferred to plates with CM4 + 5G medium (CM4 medium with the selection agent glufosinate ammonium at 5 μg/mL) and incubated for 2 weeks in the dark at RT. They were then transferred to plates with CM4 + 10 G medium (CM4 medium with glufosinate ammonium at 10 μg/mL) and incubated for 2 weeks in the dark at RT. Calli were then transferred to fresh CM4 + 10 G medium and incubated another 2 weeks under the above conditions. Calli clumps were transferred to MSP + 10G shoot production medium (MSP medium with glufosinate ammonium at 10 μg/mL) and incubated in a growth chamber with 20°C/18°C day/night temperature, a 16 h photoperiod, a light intensity of 80 μmol m^{-2} s^{-1}, and 30% relative humidity. After 2 weeks, callus clumps were transferred to plates with root production medium MSE + 5G medium (MSE medium with glufosinate ammonium at 10 μg/mL) and incubated in a growth chamber with the same conditions described before. The regenerated shoots were transferred independently to tubes with MSE + 5G and kept under the growth chamber conditions until they established a radical system and reached approximately 7 cm in length. Developed plantlets were transferred to soil in square Jiffy pots and acclimatized for 15 to 20 days in a growth chamber (20°C/15°C day/night temperature and 16 h photoperiod). Identification numbers were assigned to the shoots transferred to soil (consecutive numbers of the Plant transformation laboratory record). Vigorous plantlets were transferred to large pots and kept under the growth chamber conditions until the presence of the transgenes was evaluated.

Identification of positive transgenic plants

The presence of *bar* gene was tested using the "painting" method described in Tian et al. (2019). When the plants reached the three-leaf or four-leaf stage, one leaf of each tiller (avoiding the flag leaf) was painted with a cotton ball soaked in a 0.2% Liberty® solution (Liberty® 280 SL

herbicide, Bayer CropScience, Raleigh, NC, USA). Plants were kept under growth chamber conditions and the response to the herbicide was evaluated 7 to 10 days later. Tillers that did not show necrosis on the painted leaf were considered positive for the presence of the *bar* gene.

DNA was isolated from putative positive plants (tillers) using the DNeasy® plant mini kit (QIAGEN, Germantown, MD). The presence of the AMP genes and *bar* was evaluated by PCR. In addition, the wheat *tubulin* gene was amplified as control. Primers to amplify the AMP genes were designed based on the codon-optimized sequence of the AMPs. All the primers used in this study were synthesized by IDT (Integrated DNA Technologies, Coralville, Iowa) and the sequences are shown in the Table 2.1. PCR reactions were done following these conditions: 1X GoTaq® Flexi buffer, 2.5 mM $MgCl_2$, 400 μM each dNTP, 0.4 μM each primer, 1 unit of GoTaq® Flexi DNA polymerase (Promega, Madison, WI), and 25 ng genomic DNA in a final volume of 25 μL. The amplification program was 94 °C for 5 min, 35 cycles of 94 °C for 30 seconds, 58 °C for 30 sec, and 72 °C for 45 sec, with a final extension at 72 °C for 10 min. All reactions were completed on a Multigene Gradient thermal cycler (TC9600G, Labnet, Edison, NJ). PCR products were analyzed by electrophoresis in agarose gels (1.0 % agarose in 1X TAE electrophoresis buffer). The T_0 plants in which the presence of the AMP gene was confirmed by molecular analysis were kept under growth chamber conditions until the seeds were obtained. Seeds from individual transgenic T_0 tillers were harvested independently. Plants/seeds were labeled using the abbreviation of the plasmid used (Ace1, Wj1, ARA1 or Zma), followed by the number assigned to the shoot, and followed by a letter representing the tiller. Numbers added after the letter represent the particular identification of each individual of the next generation (T_1, T_2, T_3, etc).

Expression of AMP genes in positive T_0 plants, relative expression and determination of the transgene copy number

Expression of the transgenes was evaluated in positive T_0 plants/tillers. Tissue was collected (aproximately 100 mg of leaf tissue, ≈ 8 cm foliar lamina) in 2.0 mL microcentrifuge tubes containing two 4.5 mm beads (BBs, Daisy®, Rogers, AR) and flash frozen in liquid nitrogen. Tissue was ground in a TissueLyser (QIAGEN, Germantown, MD), alternating homogenization for 25 sec at 25 Hz and freezing in liquid nitrogen, for a total of 3 homogenizations. The ground tissue was stored at -80 °C or used directly to extract the RNA. Total RNA was isolated using 1 mL TRIzol® reagent (Invitrogen, Carlsban, CA) following the manufacture's protocol. The RNA pellet was resuspended in 100 µL of RNase-free water, quantified by NanoDrop (ThermoScientific, Waltham, MA) and stored at -80°C. Single-stranded cDNA was synthesized by reverse transcription from 1 µg of total RNA using the Reverse Transcription system with Oligo$(dT)_{15}$ Primer (Promega, Madison, WI). The reverse transcription reaction was done according to manufacturer's instructions, incubating the reaction at 42°C for at least 1 h. After the reverse transcription, 4 µL of cDNA were used as a template in conventional PCR (Reverse Transcription-PCR or RT-PCR). Amplification was completed following the conditions previously reported to identify transgenic plants, using the specific primers for each AMP gene and *bar*. *Tubulin* primers (Tub-F and Tub-R) were used to test contamination with genomic DNA (gDNA), since the PCR products obtained from gDNA are 500 base pairs (bp), while the products obtained from cDNA are 408 bp. Expression of the AMP genes was also evaluated in heads. For this, total RNA was isolated using the RNeasy mini kit (QIAGEN, Germantown, MD) according to the manufacture's recommendations. cDNA synthesis and RT-PCR were conducted following the same procedures described for leaf tissue.

The relative expression of the AMP genes in T_0 plants was determined by a two-step quantitative Real-Time PCR (qPCR) assays, using cDNA synthesized in a previous reaction (Reverse Transcription-quantitative PCR or RT-qPCR). Primers for the amplification of each AMP gene were designed based on the codon-optimized sequence, while primers for the reference gene *actin* were designed using the GeneBank accession Q5EWZ1 (Table 2.1). The optimum annealing temperature (T_a) for each set of primers was established through a thermal gradient assay with a range of temperatures: 56.6, 58.2, 60.1, and 61.7 °C. For these assays, dilutions of the cDNA (1:4 with RNase-free water) were used as template and each sample was run in triplicate. The qPCR conditions used were: 1X SsoAdvanced universal SYBR® Green supermix (Bio-Rad, Hercules, CA), 0.35 µM for each primer, and 5 µL diluted cDNA, in a 20 µL final volume. The cycling protocol for the gradient assay were: polymerase activation/denaturation at 95°C for 30 sec, 40 cycles with denaturation at 95°C for 15 sec and annealing/extension at each temperature for 30 sec, and a melt curve analysis with 65°C to 95°C, with 0.5°C increments each 5 sec. All the reactions were completed using the Bio-Rad CFX96 Real-Time System, and the data were collected using the CFX Maestro™ Software (Bio-Rad, Hercules, CA). The efficiency of each set of primers was verified by running standard curves using the optimized Ta. Serial 10-fold dilutions were prepared using a positive control sample and each dilution was run in triplicate. The reaction conditions and amplification program described above were used for the standard curves. Sets of primers that showed a percentage of efficacy between 90 and 110% and a coefficient of determination (R^2) > 0.98 for the standard curve were used in the following experiments. The relative expression of the AMP genes in the T_0 positive plants was determined using the standardized RT-qPCR conditions. Three technical replicates were run for each sample and the relative expression was calculated considering the

expression level of the reference gene (*actin*) and the expression level of the target gene (AMPs), according to the formula: Expression = $2^{\Delta Ct} = 2^{(Ct\ actin) - (Ct\ target)}$

Collier et al. (2017) reported the use of droplet digital PCR (ddPCR) as a simple and accurate approach to measure the transgene copy number in a range of crops, including wheat. Primers were designed for each AMP gene following the recommendations of the Droplet Digital™ PCR Application Guide (Bio-Rad). The wheat *Puroindoline-b* (*Pinb*) gene present as two copies in the genome (5D chromosome) was used as reference gene. Primers to amplify the *Pinb* gene (accession DQ363914) were published by Collier et al. (2017). A gradient PCR with a range of annealing temperatures from 54°C to 62°C was run to determine the optimal annealing temperature (T_a) for each set of primers. ddPCR reactions were set using 1X QX™ ddPCR™ EvaGreen supermix (Bio-Rad, Hercules, CA), 140 nM each primer, 3 units *HindIII*-HF (New England BioLabs, Ipswich, MA) and 37.5 ng DNA, adjusting the volume to 20 µL with RNase-free water. The reaction was stored overnight at 4°C and allowed to reach room temperature before generating the droplets. The droplets were generated in a QX200 Droplet Generator (Bio-Rad, Hercules, CA) according to manufacturer's instructions. Droplets (40 µL) were carefully transferred to a 96 well plates and amplification was completed in a C1000 Touch thermal cycler (Bio-Rad, Hercules, CA) with the following conditions: enzyme activation at 95°C for 5 min, 40 cycles with denaturation at 95°C for 30 sec and annealing/extension for 1 min, signal stabilization at 4°C for 5 min and 90°C for 5 min; with a ramp rate of 2°C/sec. The annealing temperature corresponded to the range of temperatures selected for the gradient (54, 54.6, 55.7, 59, 60.5, 61.5 and 62°C). When the amplification was completed, the plate was transferred to the QX200 Droplet Reader. After the droplets were read, the data was analyzed with the QuantaSoft™ Software. The optimum annealing temperature for each set of primers was that in

which the greatest number of positive droplets was generated, and the separation of positive and negative droplets was clearly defined. To measure the transgene copy number in the T_0 plants, the samples were amplified following the methodology described above, using the optimum temperature identified for each set of primers. Each sample was evaluated in two reactions, one with primers for the corresponding gene of interest and another with primers for the reference gene (*Pinb*). The concentration of each gene (copies/µL) was estimated with the QuantaSoft™ Software and the transgene copy number was calculated according to the formula: [transgene concentration (copies/µL) / reference concentration (copies/µL)] x 2.

Bioassays

Five independent experiments were completed. Transgenic plants from different generations (T_1, T_2, T_3 or T_4) were used in the assays, including two controls: Bobwhite_"wild type" and a Bobwhite transgenic line carrying and expressing the *bar* gene. Plants used in the bioassays were grown in chambers with 18°C/15°C day/night temperature, 16 h photoperiod, light intensity of 450 µE m^{-2} s^{-1}, and 50 to 60% relative humidity. Before being challenged with *F. graminearum*, the presence of the transgenes and their expression were confirmed in the plants. The presence of the transgenes was tested by direct amplification with the KAPA3G Plant PCR kit (Kapa Biosystems, Indianapolis, IN). Briefly, when the plants were in the 3- or 4-leaf stage, a piece of leaf lamina (approximately 0.5 cm x 0.5 cm) was cut with scissors and transferred to a microcentrifuge tube (1.5 ml) containing 100 µL of extraction buffer (50 mM Tris-HCl pH 8.0, 0.1 mM EDTA pH 8.0, 2% β-mercapto-ethanol). The tissue was crushed with a micropipette tip and preserved on ice. Tubes were incubated at 95°C for 5 minutes and put back on ice. The crude extract was diluted at 1:10 with double-distilled water (ddH_2O) and used fresh in PCRs. The PCR conditions were 1X KAPA PCR buffer, 1 mM $MgCl_2$, 0.3 µM each primer, 1

unit KAPA3G polymerase, 5 μL of diluted crude extract, in a final volume of 25 μL. The amplification program was 95°C for 6 minutes, 40 cycles of 95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 45 seconds, and a final extension at 72°C for 10 minutes. PCRs were completed in a Multigene Gradient thermal cycler (TC9600G, Labnet, Edison, NJ). RT-PCR was used to evaluate the expression of the transgenes in plants that were positive for transgene detection, following the methodology described before. Transgenic plants and controls were inoculated when they began anthesis (Feekes growth stage 10.5.1). One floret of the tenth spikelet, from the base of the head, was inoculated with 10 μL of the conidial suspension (100 conidia/μL), by placing the suspension in the floral cavity between the palea and lemma with a micropipette. Inoculated heads were immediately covered with black resealable plastic bags (7.6 x 12.7 cm, Ref. S-12322BL, ULINE, Coppell, TX) previously moistened with water inside. Bags were removed after 48 h. Inoculations were done every other day, by selecting the plants that were in the appropriate developmental stage (anthesis), until all the plants were inoculated. FHB disease severity was determined as the percentage of spikelets with disease symptoms (PSS), considering the 10 spikelets at the base of the head. For all the experiments, the area under disease progress curve was calculated using the trapezoidal method proposed by Madden et al. (2007). Analysis of variance (ANOVA) of the variable AUDPC were done using the GLM procedure in the SAS software, Version 3.8, Enterprise Edition. Copyright© 2012-2018 SAS Institute Inc (Cary, NC).

The specific conditions of each of the experiments are presented below: ***Bioassay 1****:* this experiment was conducted with Dr. Willian W. Bockus (Department of Plant Pathology, Kansas State University) during June 2016. Six plants per transgenic line and controls were used, 3 heads per plant were inoculated using a conidial suspension of the *F. graminearum* isolate 3636.

This experiment was completed under greenhouse conditions with a 32°C/21°C (day/night) temperature. The PPS was scored 10, 12 and 14 days after inoculation (dai). For the following experiments the plants were kept under growth chamber conditions (20°C/18°C day/night temperature, 16 h photoperiod, light intensity of 400 μE m^{-2} s^{-1}, and a relative humidity of 60%) and the inoculations were made with a conidial suspension of *F. graminearum* (100 conidia/μl) provided by the laboratory of Dr. Gui-Hua Bai (USDA/ARS, Department of Agronomy, Kansas State University). ***Bioassay 2****:* eight plants per transgenic line and controls were used, 3 heads per plant were inoculated. PPS was evaluated 10, 12 ,14 and 16 dai. ***Bioassay 3***: ten plants per transgenic line and controls were used, 3 heads per plant were inoculated. PPS was scored every other day, from 6 to16 dai. ***Bioassay 4****:* twelve plants per transgenic line and controls were used, 3 heads per plant were inoculated. PPS was evaluated every other day, from 6 to 16 dai. ***Bioassay 5****:* twelve plants per transgenic line and controls were used, 2 heads per plant were inoculated. PPS was evaluated every other day, from 6 to 16 dai.

Results

Recovery and molecular characterization of transgenic plants

Ten independent biolistic transformation experiments were performed for the *Ace-AMP1* gene. A total of 1375 embryogenic calli were used in these experiments. Sixty-three plants were recovered after acclimatization and they were "painted" with the herbicide. Ten plants showed resistance to the herbicide, and the molecular analysis confirmed the presence of the *bar* gene in 9 of these plants, but only 8 of them also carried the *Ace-AMP1* gene. The transformation efficiency for this gene was 0.58%.

For the *WD* gene, twelve independent bombardment experiments were performed, using a total of 1600 embryogenic calli. Sixty-three plants were tested with the herbicide, and 14 plants

showed resistance. Molecular analysis confirmed that the 14 herbicide-resistant plants carried the *bar* gene, but the *WD* gene was only detected in 8 plants. The transformation efficiency was 0.5%.

A total of 1225 embryogenic calli were bombarded with the plasmid carrying the *ARACIN1* gene, in 9 independent experiments. Seventy-four plants were painted, and 13 of them were resistant to the herbicide. The presence of the *bar* gene was confirmed by PCR in 12 of these herbicide-resistant plants, and *ARACIN1* was detected in 10 of them. The transformation efficiency for *ARACIN1* was 0.82%.

Fourteen independent transformation experiments were conducted for the *Zeamatin* gene, using a total of 1425 embryogenic calli. After acclimation, 39 plants survived and were tested with the herbicide. Eight plants were resistant to the herbicide and presence of the *bar* gene was confirmed in 7 of these plants by PCR; however, the *Zeamatin* gene was only detected in 6 of them. The transformation efficiency for this gene was 0.42%.

Evaluations made by RT-PCR established that all T_0 positive plants were expressing the corresponding AMP gene. The expression in heads was also positive for all the plants evaluated. The levels of expression of the transgenes, determined by RT-qPCR, showed high variation between the T_0 plants carrying and expressing the same transgene (Figure 2.1). Measurements of the copy number estimated by ddPCR were close to integer values in most cases (27 out of 32 samples analyzed), indicating that the methodology was useful to estimate the copy number in these samples. However, the copy number was estimated to be mid-way between integers (i.e. between .4 and .6) in 5 samples: Ace1_8666.A, Wj1_8582.A, ARC1_8541.C, Zma_8558.A, and Zma_8796.C, therefore assigning a copy number to these samples was not possible. The copy

number was estimated for the sample Zma_8738.A, but tissue could not be recovered to isolate total RNA because the plant dried prematurely.

T_1 seeds were recovered from most of the T_0 plants, but plants Ace1_8618, ARA1_8718, and Zma_8738 were sterile or dried prematurely. Transgenic lines were advanced until T_3 or T_4 generations and segregation of the transgene (progeny without the AMP gene) was frequently observed. For this reason, it was necessary to check the presence of the transgene in all the plants that were used in the bioassays.

Bioassays

Plants from twenty transgenic lines were challenged with *F. graminearum*, 5 Ace1 lines, 5 Wj1 lines, 7 ARA1 lines, and 3 Zma lines). The responses of plants to FHB, measured as a percentage of spikelets with symptoms (PSS) throughout the evaluation period, are presented in Figure 2.2; the AUDPCs calculated for each line are presented in the Figure 2.3.

In the ***Bioassay 1***, T_1 plants from 8 transgenic AMP lines were tested; only the line Ace1_8666.A showed a reduction in the percentage of spikelets with symptoms (Figure 2.2.A). This was reflected in a decrease in the area under the curve, which was significantly different from that of other transgenic lines and controls evaluated (Figure 2.3.A). In the ***Bioassay 2***, T_2 plants from 3 AMP lines were tested, but significant differences in the responses were not observed (Figure 2.3.B). In this assay, the line Wj1_8538.C.2 showed a slight reduction in the percentage of symptomatic spikelets during the first days of evaluation (Figure 2.2.B). Five Wj1 lines were challenged with FHB in the ***Bioassay 3***. Lines Wj1_8556.A.4.1 and Wj1_8582.A.3 had lower percentages of spikelets with FHB symptoms (Figure 2.2.C) and significant differences in AUDPCs (Figure 2.3.C) compared to other transgenic lines and controls. In this assay, the percentage of spikelets affected was low overall, and even the control Bobwhite_WT

showed an average of 77.92% infection on the last evaluation day (16 dai). T_3 plants from line Wj1_8538.C.2.15, derived from line Wj1_8538.C.2, were tested in the ***Bioassay 3***, but showed no reduction in the response to FHB. On the contrary, they were highly susceptible (Figure 2.2.C and Figure 2.3C). In the ***Bioassay 4***, four AMP lines were tested. aT_2 plants from the line ARC1_8894.D.1 consistently showed a low percentage of infected spikelets during all evaluation days (Figure 2.2.D), and statistical analysis confirmed the differences in the AUDPC (Figure 2.3.D). Plants from five AMP lines were tested in the ***Bioassay 5***, including T_3 plants from the line ARC1_8894.D.1.4 derived from ARC1_8894.D.1. The T_3 plants of the line ARC1_8894.D.1.4 showed a low percentage of spikelets affected over time and the AUDPC showed significant differences when compared to other materials (Figure 2.2.E and 2.3.E). A comparison of heads of three plants ARC1_8894.D.1.4.4, Zma_8558.A.5.4.12 and Bobwhite_WT.5, inoculated with *F. graminearum* on three different evaluation days are shown in Figure 2.4. Most of the transgenic plants of the line ARC1_8894.D.1 showed a distinctive spike morphology. These spikes were more slender (elongated and thin) than typical spikes observed in other transgenic lines and in Bobwhite_wild type (Figure 2.5), although they did not show any difference in their development, such as emergence time or flowering time. Statistical analysis revealed that the AUDPC of lines Ace1_8866.A (T_1), Wj1_8556.A.4.1 (T_3), Wj1_8582.A.3 (T_2), ARC1_8894.D.1 (T_2 and T_3) were significantly different from those of other transgenic lines and controls, but the mean percentages of spikelets with symptoms recorded on the last day of evaluation (14 or 16 dai) were high, ranging between 68 and 86 % (Figure 2.3), and the reduction in the percentage of spikelets with symptoms ranged between 7.9 and 15%, when compared to controls. Therefore, plants of these transgenic lines are considered as susceptible to FHB.

Discussion

FHB resistance is a quantitative trait controlled by multiple major and minor quantitative trait loci (QTLs) (Bai et al., 2018), which has made the breeding of commercial cultivars slow and complex. Because of this, a biotechnological approach was used in this study as an attempt to reduce the susceptibility of wheat to FHB. Four genes encoding the plant antimicrobial peptides (AMPs), *Ace-AMP1*, *WD*, *ARACIN1*, and *Zeamatin*, were independently introduced and expressed in the susceptible spring cultivar 'Bobwhite'.

Considering all the transformation experiments, more than 5600 embryogenic calli were co-bombarded with two DNA plasmids, one carrying *bar*, the selectable marker gene that confers resistance to the herbicide glufosinate, and a plasmid carrying the gene of interest (GOI, in this study, each of the AMP genes). Forty-two T_0 plants with the *bar* gene were identified, but the AMP gene was only detected in 32 of these plants, which means that the co-transformation frequency was 76%. In a previous study, in which 70 independent transgenic wheat plants generated by particle bombardment and their progeny were analyzed, it was found that 85% of the transformed plants contained the selection gene and the GOI (Stoger et al., 1998). Similarly, Tian et al. (2019) reported that 90% of transgenic wheat plants contained both genes when embryogenic calli were used as explant in biolistic experiments. The high co-transformation frequencies observed can be explained by the formation of transgene clusters. It has been proposed that the co-integration of genes in rice results from a two-phase integration mechanism, first the transgenes are ligated forming continuous arrays (plasmid-plasmid junctions); in the second step, these arrays are integrated in the same locus, forming clusters, interspaced by short regions of genomic DNA (Kohli et al., 1998; Kohli et al., 1999). This type of tandem integrations was also observed in wheat, where transgenes from co-transformation experiments

were inserted as tandem repeats interspersed with unknown DNA (Jackson et al., 2001). Considering the four genes, the average transformation efficiency was 0.58%, which is much lower than the transformation efficiency reported (5 – 10%) by Tian et al. (2019) using the same biolistic protocol. In the expression analysis of the transgenic T_0 plants using RT-PCR , it was confirmed that all the plants that carried *bar* and the AMP gene, co-expressed both genes, and transgene silencing was not observed in T_0 plants. The high co-expression of both transgenes was previously reported by Stoger et al. (1998), who found co-expression frequencies close to 90%. Co-segregation of *bar* gene with the GOI (AMP genes) was observed in the T_1 and following generations. Although transgene loss was observed in some individuals of the T_2, T_3 and T_4 generations, transgene silencing was not observed in any of the plants that carried the transgenes. This is contrary to Anand et al. (2003a) observations, who found that at least the selection gene was silenced in the T_1 progeny of 18 of the 24 primary transgenic lines. The authors determined that this silencing was due to methylation of the maize *Ubiquitin1* promoter used in their experiments (Anand et al., 2003a). In this study, the copy number of the GOIs was measured using ddPCR (droplet digital PCR) in the 32 positive T_0 plants. The copy number ranged from one copy to 18 copies, where 12% of the plants had a single copy, 38% had 2-3 copies, 16% had 4-5 copies, and 34% had more than 5 copies. These results varied compared to some previously published studies where particle bombardment was also used to generate transgenic wheat. In those studies, where the copy number was determined using Southern blotting, the authors found a high percentage of plants with a single copy (almost 25%), while the percentage of plants with more than 5 copies was low (around 12%) (Blechl et al., 1998; Rasco-Gaunt et al., 2001). The relative expression analysis of the AMP genes in the 32 T_0 transgenic plants showed high variation among plants generated using the same transgene

constructs (Figure 2.1). Several factors may be responsible for the variation in the expression of transgenes in plants, including copy number of the transgene, location of the integration site, and effects of DNA methylation (Butaye et al., 2005). Regarding transgene copy number, the expression has been associated with high number of copies, with low number of copies, or simply not associated. For example, in transgenic rice plants no correlation was observed between transgene copy number and the level of expression (Maqbool & Christou, 1999); the same was reported for transgenic plants of *Nicotiana plumbaginifolia* (Shirsat et al., 1989). Stoger et al. (1998) reported that the highest expression levels were found in transgenic wheat plants containing multiple transgene copies. In some cases, the type of promoter used may have been the most critical factor affecting transgene expression. For example, Jackson et al. (2001) reported that neither the copy number, nor integration type, nor the locations of the integration site affected the expression of transgenes in wheat, when a constitutive promoter, like *ubiquitin* promoter, was used. Chen et al. (1999) also reported the silencing of genes controlled by the CaMV 35S promoter, while genes under the control of *Ubiquitin* promoter were expressed until the T_3 generation. In this study, correlation between the number of copies of the transgene and the level of expression was not observed, and all transgenes (*bar* and AMP genes) were under the control of the *Ubi1* promoter. Therefore, it is likely that the differences in expression levels observed in T_0 plants were due to the effects of the integration site.

The four peptides evaluated in this study, Ace-AMP1, WD, ARACIN1, and Zeamatin, were selected because they demonstrated *in vitro* or *in vivo* antifungal activity in previous reports (Cammue et al., 1995; Kanzaki et al., 2002; Khan et al., 2014; Kong et al., 2014; Li et al., 2003; Malehorn et al., 1994; Mohandas et al., 2013; Neukermans et al., 2015; Ntui et al., 2010; Patkar & Chattoo, 2006; Roberts & Selitrennikoff, 1990; Roy-Barman et al., 2006; Saitoh et al., 2001;

Wu et al., 2011). Of the 20 transgenic lines evaluated for susceptibility to *F. graminearum* (5 Ace1 lines, 5 Wj1 lines, 7 ARA1 lines, and 3 Zma lines), only 4 lines showed a slight reduction in the percentage of spikelets with symptoms (PSS) and/or a delay in the development of symptoms: Ace1_8866.A, Wj1_8556.A.4.1, Wj1_8582.A.3, and ARC1_8894.D.1. However, plants from these transgenic lines were considered susceptible because at the end of the evaluation period (14 or 16 dai), the PSS was close to or greater than 70%. Particularly, T_2 and T_3 plants from the line ARC1_8894.D.1 showed a delay in the development of symptoms throughout the entire evaluation period (6 to 16 dai), and the highest reduction in the severity on the last day of evaluation (17 and 12% reductions in PSS at 16 dai, compared to Bobwhite_WT, in T_2 and T_3 plants, respectively). Plants of the ARC1_8894.D.1 line presented a slender morphology of their spikes, not observed in any other line expressing *ARACIN1* gene. Therefore, it is possible that this phenotype is more related to the site of transgene integration in the host genome than with the transgene. A slower development of FHB symptoms in transgenic plants expressing AMP genes or pathogenesis-related (PR) protein genes has been previously reported. Chen et al. (1999) reported that T_1, T_2 and T_3 plants from a transgenic wheat line expressing a rice thaumatin-like protein gene (*tlp*) showed a slower FHB symptom development than control plants during the first days of evaluation (10 to 14 dai), but after 3 weeks, spikes from transgenic and control plants were wilted. A transgenic wheat line over-expressing the wheat *chitinase* and *β-1,3-glucanase* genes showed a delay in the progression of the FHB infection under greenhouse conditions, but the line was susceptible in field experiments (Anand et al., 2003b). Successful examples where the expression of AMPs in transgenic wheat plants has resulted in enhanced resistance to FHB have also been described. Expression of RsAFP2, a plant defensin from seeds of radish, in transgenic wheat plants resulted in enhanced resistance to FHB, and this resistance

was observed until the T_5 generation in greenhouse and field experiments (Li et al., 2011). Badea et al. (2013) results showed that expression of the synthetic peptides MsrA2 and 10R in transgenic wheat plants resulted in an average reduction of 50% in FHB susceptibility when compared to controls. Unfortunately, it is not clear if the resistance conferred by these AMPs resulted from a direct action on the pathogen, or because the peptides were having an effect on signal transduction cascades related with defense. The role of plant AMPs in the modulation of the defense response to pathogens has been widely referenced (Bolouri Moghaddam et al., 2016; Campos et al., 2018), however, for three of the peptides evaluated in this study, this type of activity has not been reported. A high accumulation of salicylic acid (SA) and greater or faster expression of defense-related genes like *PAL* (phenylalanine ammonia lyase), *PR2* (β-1, 3-glucanase) and *PR3* (chitinase) were observed in transgenic wheat heads expressing *Ace-AMP1* inoculated with *Neovossia indica* (karnal bunt), than in inoculated null-segregant control heads (Roy-Barman et al., 2006). Indirect evidence of AMPs activity in transgenic wheat plants could be obtained by assessing the levels of expression of genes related with the basal defense in wheat. Possible changes in the expression would not only confirm the putative role of AMPs in the regulation of this response but, would indicate that active forms of peptides are being synthesized in the wheat transgenic plants.

The role of pathogenesis-related proteins (PR), jasmonic acid (JA)-, salicylic acid (SA)- , and ethylene (ET)- signaling pathways in the resistance to FHB has not been fully clarified. Studies aimed at clarifying the response of wheat to *F. graminearum* infection have been performed using microarrays (Affimetrix GeneChip Wheat Genome Array) and transcriptome sequencing, evaluating the response of the resistant cultivar Sumai 3, and the Chinese landrace Wangshuibai, both used as sources of resistance, respectively (Li & Yen, 2008; Xiao et al. 2013).

In both studies it was found that the response of resistant materials is associated with an over-expression of genes related to JA-signaling pathway. Xiao et al. (2013) also reported upregulation of some genes related with PR3 (chitinases), PR5 (thaumatin-like proteins), and PR14 (non-specific lipid transfer proteins) in Wangshuibai; whereas Li and Yen (2008) did not report any PR gene associated with resistance in Sumai3. Li and Yen (2008) suggested that together with JA-signaling pathway, the ET pathway is involved in the resistance of Sumai3. Both studies concluded that neither PR1 nor SA-mediated defense pathways are involved in the resistance. These results are opposed to those presented by Makandar et al. (2006), and Makandar et al. (2012), who suggested that SA regulates resistance to FHB in wheat. The over-expression of the *A. thaliana NPR1* gene (regulator of SA signaling) in transgenic wheat resulted in a fast and high accumulation of wheat PR1 and in the restriction of the pathogen in inoculated spikelets (Makandar et al., 2006). Makandar et al. (2012) showed that applications of SA, as a soil drench, induced PR1 expression and enhanced resistance to FHB, whereas disease severity was high in transgenic plants expressing the NahG gene, which encodes an SA-degrading salicylate hydroxylase.

In this study, the expression of the genes encoding antimicrobial peptides in transgenic plants was confirmed at the messenger RNA (mRNA) level, but translation into proteins was not proven due to the absence of antibodies raised against these AMPs or fused tags (e.g. 6X-his tag or FLAG-tag) that allowed detection or purification of the proteins. An indirect alternative to assess that transgenic plants are synthesizing biologically active peptides would be to perform *in vitro* antifungal activity tests. Crude protein extracts could be obtained from leaves of transgenic plants, and protein fractions with the expected sizes could be concentrated through membrane filtration. These concentrated proteins could then be used in microplate inhibition assays

(Broekaert et al., 1990; Cavallarin et al. 1998) to evaluate the possible activity against *F. graminearum* and other fungal species, on which the antifungal activity of these AMPs was already confirmed.

Although the expression of AMPs did not confer high levels of resistance to FHB, the transgenic lines that showed reduction in symptoms should be evaluated in more detail to determine if the expression of these genes would have an effect on the basal defense of wheat. A better understanding of the biology of the pathogen and the resistance of wheat can be valuable substrates for the implementation of new biotechnological approaches that contribute to the development of wheat lines with resistance to *F. graminearum*.

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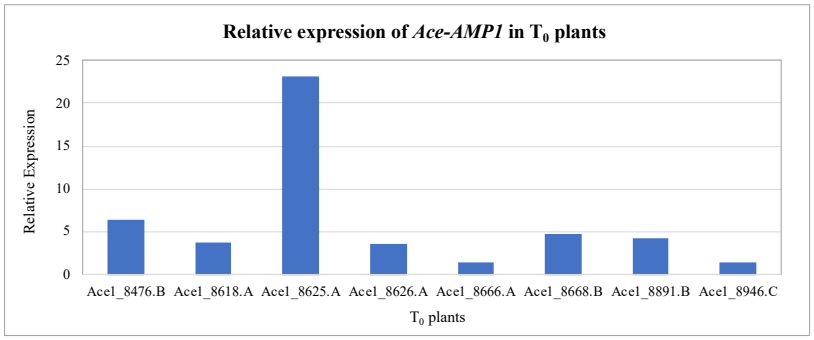
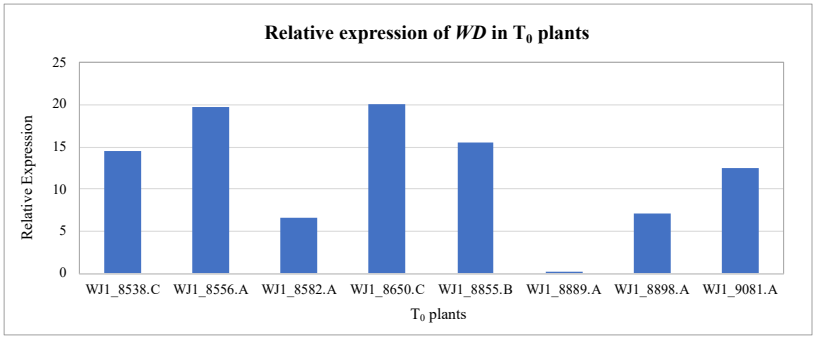
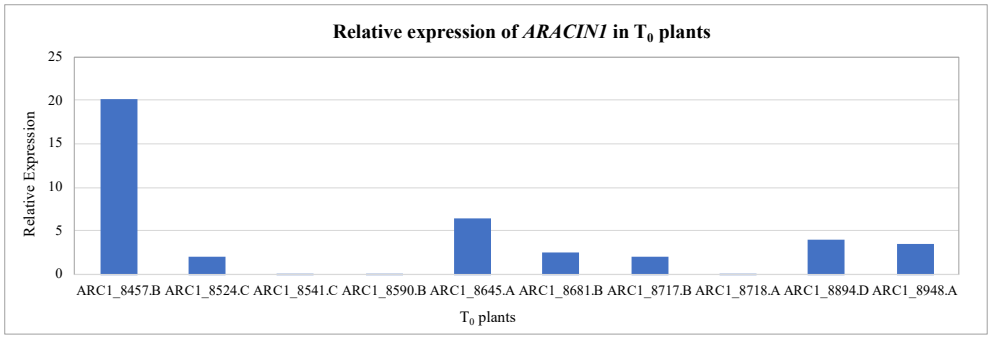


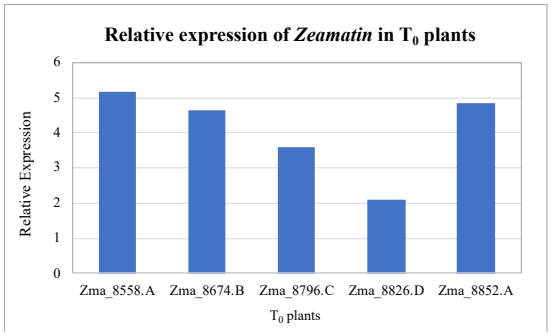
T0 plant	Estimated number of copies
Ace1_8476.B	8.03
Ace1_8618.A	1.74
Ace1_8625.A	10.79
Ace1_8626.A	3.12
Ace1_8666.A	1.40
Ace1_8668.B	3.88
Ace1_8891.B	17.81
Ace1_8946.C	2.07



T0 plant	Estimated number of copies
Wj1_8538.C	10.78
Wj1_8556.A	8.15
Wj1_8582.A	0.61
Wj1_8650.C	8.03
Wj1_8855.B	2.13
Wj1_8889.A	1.01
Wj1_8898.A	6.76
Wj1_9081.A	5.21



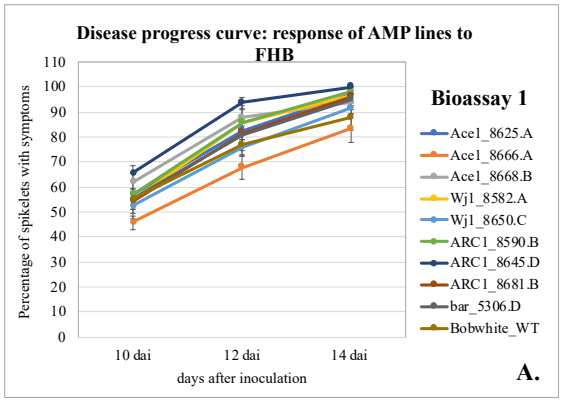
T0 plant	Estimated number of copies
ARC1_8457.B	11.10
ARC1_8524.C	3.71
ARC1_8541.C	2.37
ARC1_8590.B	2.01
ARC1_8645.A	3.01
ARC1_8681.B	3.23
ARC1_8717.B	2.91
ARC1_8718.A	0.94
ARC1_8894.D	4.96
ARC1_8948.A	8.99

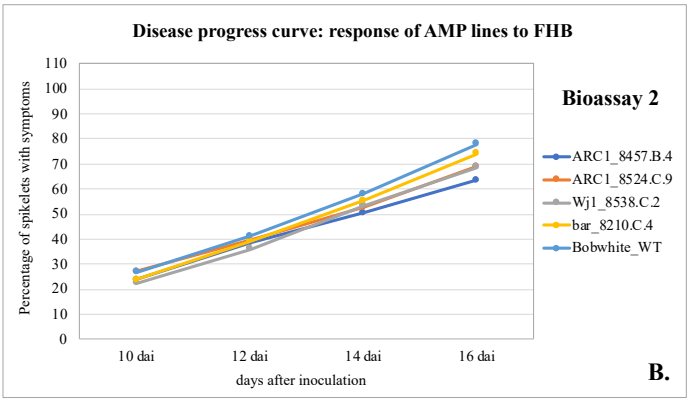


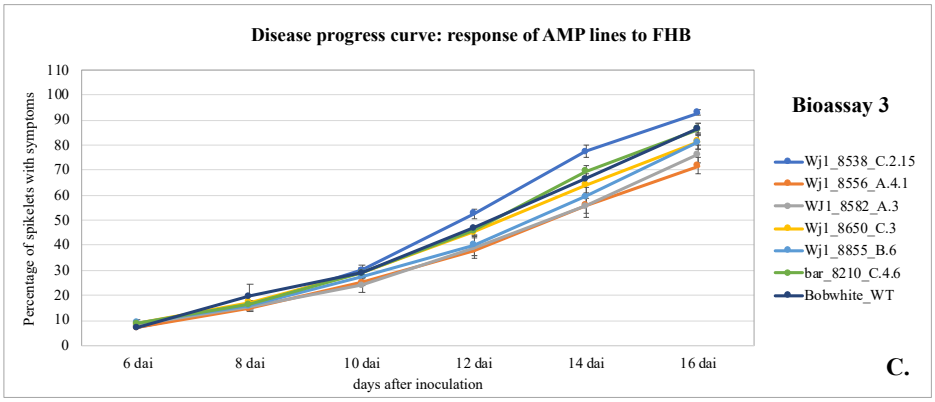
T0 plant	Estimated number of copies
Zma_8558.A	2.37
Zma_8674.B	6.29
Zma_8738.A	9.24
Zma_8796.C	2.62
Zma_8826.D	2.85
Zma_8852.A	3.94

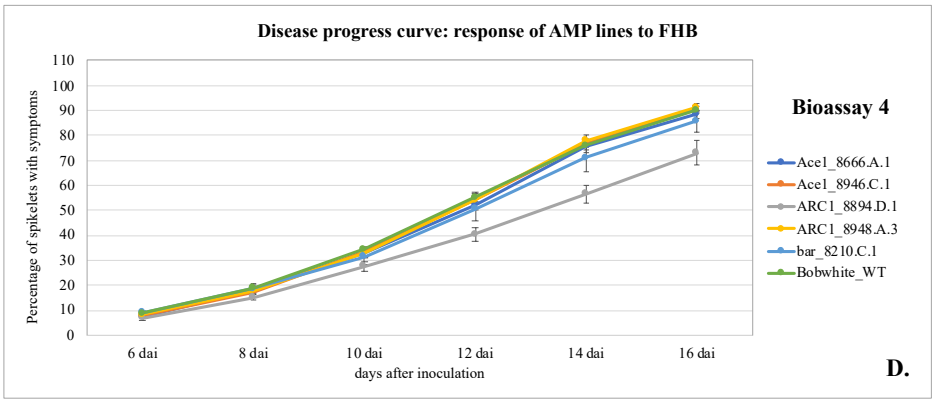
Figure 2.1 Relative expression and number of copies of the transgenes *Ace-AMP1*, *WD*, *ARACIN1*, and *Zeamatin* in T_0 plants.

Relative expression was estimated using the ΔCt method, with *actin* as reference gene; The copy number was estimated by ddPCR using *Puroindoline-b* (*Pinb*) as reference gene.


Disease progress curve: response of AMP lines to FHB
Percentage of spikelets with symptoms
days after inoculation
10 dai
12 dai
14 dai
Bioassay 1
Ace1_8625.A
Ace1_8666.A
Ace1_8668.B
Wj1_8582.A
Wj1_8650.C
ARC1_8590.B
ARC1_8645.D
ARC1_8681.B
bar_5306.D
Bobwhite_WT
A.


Disease progress curve: response of AMP lines to FHB
Percentage of spikelets with symptoms
days after inoculation
10 dai
12 dai
14 dai
16 dai
Bioassay 2
ARC1_8457.B.4
ARC1_8524.C.9
Wj1_8538.C.2
bar_8210.C.4
Bobwhite_WT
B.


Disease progress curve: response of AMP lines to FHB
Percentage of spikelets with symptoms
days after inoculation
6 dai
8 dai
10 dai
12 dai
14 dai
16 dai
Bioassay 3
Wj1_8538_C.2.15
Wj1_8556_A.4.1
WJ1_8582_A.3
Wj1_8650_C.3
Wj1_8855_B.6
bar_8210_C.4.6
Bobwhite_WT
C.


Disease progress curve: response of AMP lines to FHB
Percentage of spikelets with symptoms
days after inoculation
6 dai
8 dai
10 dai
12 dai
14 dai
16 dai
Bioassay 4
Ace1_8666.A.1
Ace1_8946.C.1
ARC1_8894.D.1
ARC1_8948.A.3
bar_8210.C.1
Bobwhite_WT
D.

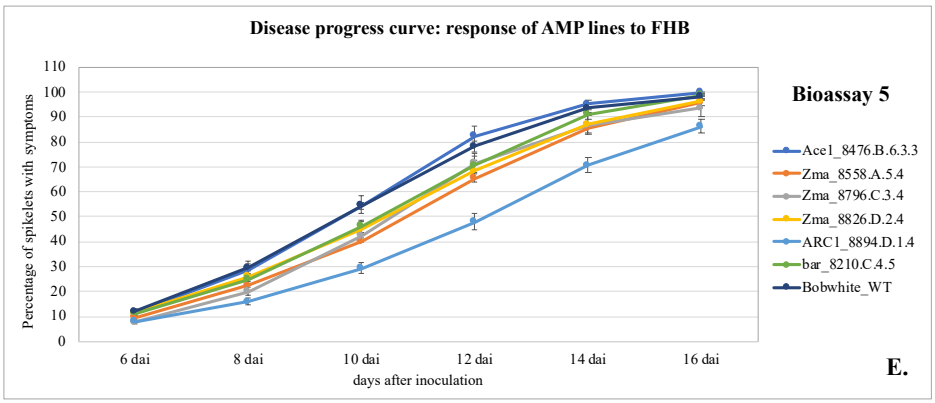
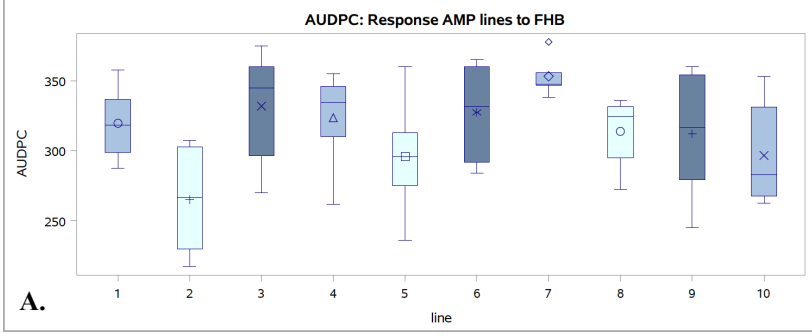

Disease progress curve: response of AMP lines to FHB
Percentage of spikelets with symptoms
days after inoculation
6 dai
8 dai
10 dai
12 dai
14 dai
16 dai
Bioassay 5
Ace1_8476.B.6.3.3
Zma_8558.A.5.4
Zma_8796.C.3.4
Zma_8826.D.2.4
ARC1_8894.D.1.4
bar_8210.C.4.5
Bobwhite_WT
E.

Figure 2.2 Disease progress curves obtained in each of the bioassays.

Figure A: bioassay 1, B: bioassay 2, C: bioassay 3, D: bioassay 4, and E: bioassay 5. The response of the plants of the transgenic lines and of the controls to FHB was evaluated as the percentage of spikelets with symptoms observed at different times (days) after inoculation. Error bars represent the standard error.

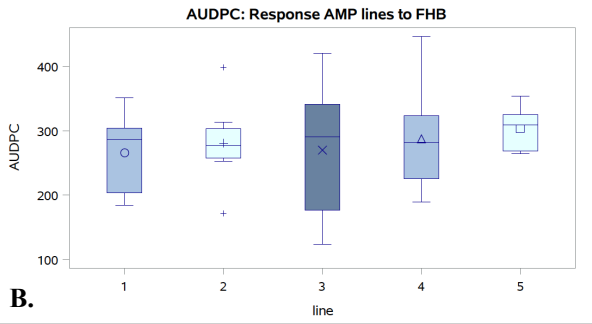


A.

Bioassay 1

	Line	14 dai	AUDPC	
1	Ace1_8625.A	97.22	319.61	ab
2	Ace1_8666.A	83.61	265.17	a
3	Ace1_8668.B	94.17	331.83	ab
4	Wj1_8582.A	96.67	323.56	ab
5	Wj1_8650.C	91.67	295.89	ab
6	ARC1_8590.B	98.33	327.36	ab
7	ARC1_8645.D	100	353.17	b
8	ARC1_8681.B	96.39	314.06	ab
9	bar_5503.E	95.28	312.08	ab
10	Bobwhite_WT	87.78	296.61	ab

P > F 0.0179

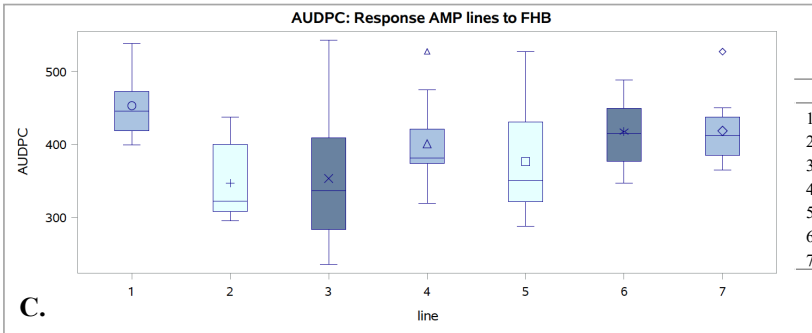


B.

Bioassay 2

	Line	16 dai	AUDPC
1	ARC1_8457.B.4	63.63	265.19
2	ARC1_8524.C.9	69.06	280.50
3	Wj1_8538.C.2	68.54	269.79
4	bar_8210.C.4	74.08	287.25
5	Bobwhite_WT	77.92	303.0

P > F 0.853

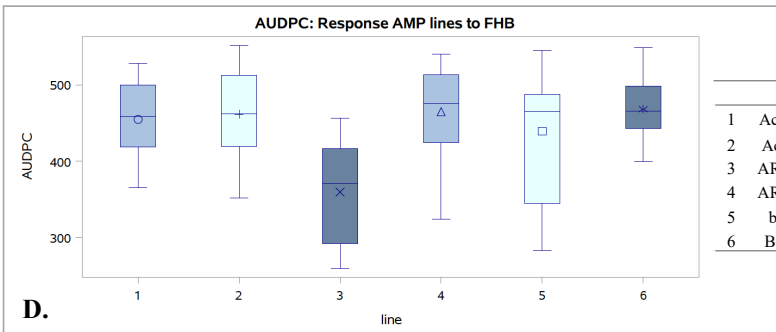


C.

Bioassay 3

	Line	16 dai	AUDPC	
1	Wj1_8538_C.2.15	93.02	452.55	b
2	Wj1_8556_A.4.1	71.73	346.80	a
3	WJ1_8582_A.3	76.18	353.15	a
4	Wj1_8650_C.3	81.38	400.70	ab
5	Wj1_8855_B.6	81.27	376.62	ab
6	bar_8210_C.4.6	86.38	417.40	ab
7	Bobwhite_WT	86.67	418.40	ab

P > F 0.0033

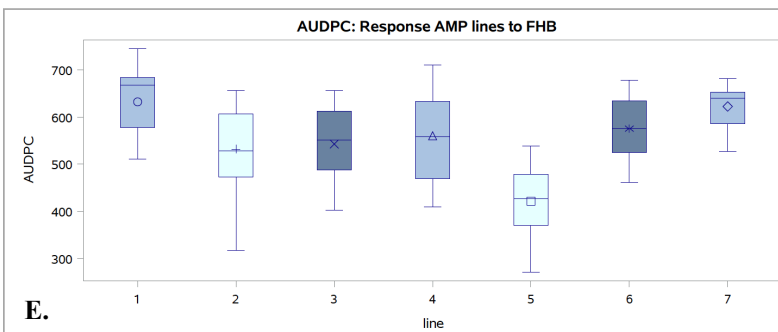


D.

Bioassay 4

	Line	16 dai	AUDPC	
1	Ace1_8666.A.1.	88.51	454.97	b
2	Ace1_8946.C.1	90.96	461.64	b
3	ARC1_8894.D.1	73.00	359.53	a
4	ARC1_8948.A.3	91.17	465.00	b
5	bar_8210.C.1	85.86	439.38	b
6	Bobwhite_WT	90.31	468.24	b

P > F 0.0021



E.

Bioassay 5

	Line	16 dai	AUDPC	
1	Ace1_8476.B.6.3.3	99.78	632.44	c
2	Zma_8558.A.5.4	95.75	531.63	b
3	Zma_8796.C.3.4	93.46	542.58	bc
4	Zma_8826.D.2.4	96.27	560.09	bc
5	ARC1_8894.D.1.4	86.25	421.67	a
6	bar_8210.C.4.5	98.63	575.25	bc
7	Bobwhite_WT	98.21	622.13	bc

P > F <.0001

Figure 2.3 Area Under Disease Progress Curve (AUDPC) calculated for the transgenic lines and controls evaluated in each of the bioassays.

Figure A: bioassay 1, B: bioassay 2, C: bioassay 3, D: bioassay 4, and E: bioassay 5, using the trapezoidal method proposed by Madden et al. (2007). ♠ mean percentage of spikelets affected scored on the last evaluation day (14 or 16 dai). ♣AUDCP means were analyzed using the GLM procedure (SAS v.3.8), with a significance level of 5% (α= 0.05). When significant differences were observed, the means were compared with the Ryan-Einot-Gabriel-Welsch F (REGWF) multiple comparison test. AUDPC means with the same letter are not significantly different.

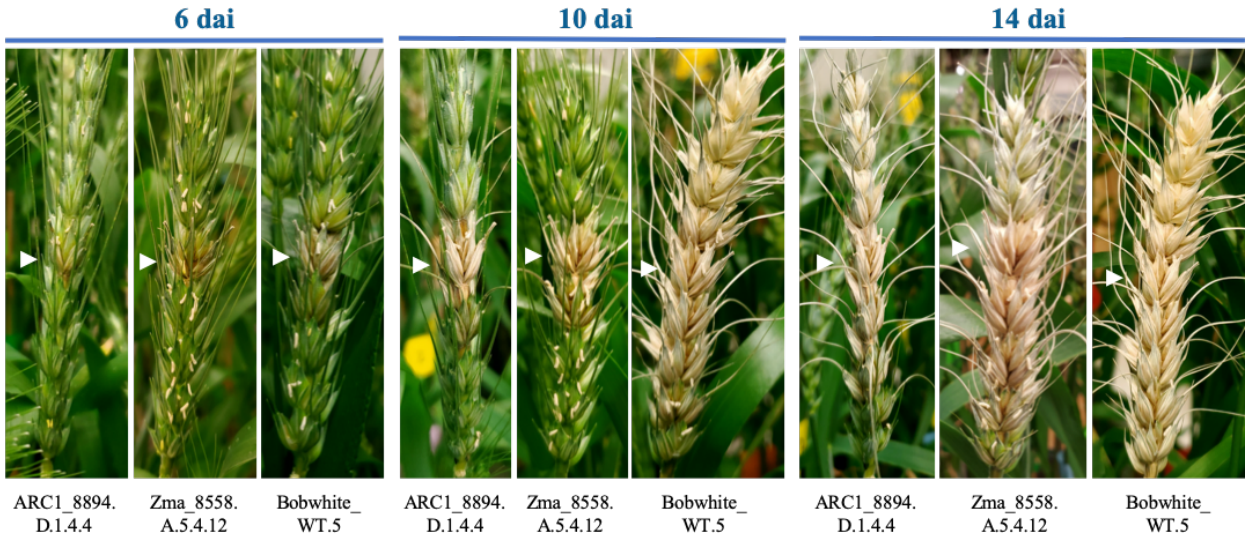


Figure 2.4 *F. graminearum* infection progress in the heads of three plants of the transgenic lines ARC1_8894.D.1.4 and Zma_8558.A.5.4 and the control Bobwhite_WT.

The white arrow points to the tenth spikelet, where the inoculum was placed.

Figure 2.5 Spike morphology observed in some plants of the transgenic line ARC1_8894.D.1, compared to the morphology of a Bobwhite_wild type spike.

Table 2.1 Primers used for detection (PCR), semiquantitative expression (RT-PCR), quantitative expression (RT-qPCR) and copy number determination (ddPCR).

Primer name	Sequence (5' - 3')	PCR product size (bp)	Description
Tub-F	ATCTGTGCCTTGACCGTATCAGG	500 gDNA / 409 cDNA	PCR and RT-PCR: control DNA contamination in cDNA samples
Tub-R	GACATCAACATTCAGAGCACCATC		
UbiABF	CCTGCCTTCATACGCTATTTATTTGC	453	PCR - *bar* detection
BarABR	CTTCAGCAGGTGGGTGTAGAGCGTG		
Bar F2	AGTCGACCGTGTACGTCTCC		RT-PCR - *bar* expression
Bar R	GAAGTCCAGCTGCCAGAAAC		
Ace-AMP1_F	GTACGCTAACAGCCAAAACATCT	319	PCR and RT-PCR - *Ace-AMP1* detection and expression
Ace-AMP1_R	ATCCAGGTAGATGAGCTTGTCC		
Wj1_F	GCTAAGTTCGCCTCGATCAT	214	PCR and RT-PCR - *WD* detection and expression
Wj1_R	GATGGTATGGGAAGATGTAGTTG		
Aracin1_F	GATGAAGACCTCCCACGTTCT	207	PCR and RT-PCR - *ARACIN1* detection and expression
Aracin1_R	GTGACGTCCTCCGAGTTCTG		
Zmatin_F	CTGTTTTCACGGTGGTCAAC	561	PCR and RT-PCR - *Zeamatin* detection and expression
Zmatin_R	CATCGTCCTTAGGGTAAGAGTAAG		
qActin_2F	AGCTGGAGACTGCCAAGAAC	124	RT-qPCR - reference gene
qActin_2R	ATCATGGATGGCTGGAAGAG		
qAce_AMP1_1F	ACAGAATCGTTACGCCTTGC	105	RT-qPCR - *Ace-AMP1* relative expression
qAce_AMP1_1R	TGAGGTTTCTGGTGTTGACG		
qWj_gth1_1F	AAGCTCTGCGAGAAGTCCAG	130	RT-qPCR - *WD* relative expression
qWj_gth1_1R	AGCGATGGTATGGGAAGATG		
qARA1_2F	TGCTCTGCCTGATGTTCG	114	RT-qPCR - *ARACIN1* relative expression
qARA1_2R	AGTTGAACTCCGCGATGC		
qZma_1F	CTAGAACCGGGTGCAAGTTC	116	RT-qPCR - *Zeamatin* relative expression
qZma_1R	GTACTCGGCGAGGGTGTTAG		
dd_Pinb_F1	AGTTGGCGGCTGGTACAATG	106	ddPCR - reference gene (Collier et al. 2017)
dd_Pinb_R1	ACATCGCTCCATCACGTAATCC		
dd_Ace1_F	ACAGAATCGTTACGCCTTGC	118	ddPCR - *Ace-AMP1* copy number determination
dd_Ace1_R	CAAGCAGCTCTCCTGAGGTT		
dd_Wj1_F	GCAAAAGCTCTGCGAGAAGT	134	ddPCR - *WD* copy number determination
dd_Wj1_R	AGCGATGGTATGGGAAGATG		
dd_ARA1_F	CAGATATCTCCACGCCACCT	113	ddPCR - *ARACIN1* copy number determination
dd_ARA1_R	GTTCTGCAGCCTTGGTCTTC		
dd_Zma_F	CTAGAACCGGGTGCAAGTTC	116	ddPCR - *Zeamatin* copy number determination
dd_Zma_R	GTACTCGGCGAGGGTGTTAG		

Chapter 3 - Prevalence of effector genes in *Magnaporthe oryzae Triticum* pathotype and biotechnological tools to enhance resistance to wheat blast

Abstract

Wheat blast is a very destructive disease with the potential to cause 100% yield loss under optimal conditions. The disease is caused by the fungus *Magnaporthe oryzae Triticum* pathotype (MoT) and was initially reported in Brazil in 1985. Wheat blast was restricted to South America for more than three decades, but now it is also found in South Asia. There are few sources of genetic resistance to wheat blast, and more virulent isolates are emerging. The goal of this study was to generate transgenic wheat plants with enhanced resistance to wheat blast. For this, the presence/absence of 22 effector genes was evaluated in a group of 103 MoT isolates from Brazil, Bolivia and Paraguay, collected between 1986 and 2017. Four avirulence (*AVR*) genes, *AVR-Piz-t*, *AVR-Pi9*, *AVR-Pi54* and *ACE1*, were detected in more than 94% of the isolates evaluated. The rice blast resistance gene *Piz-t* was used to transform wheat embryogenic calli of the susceptible cultivar 'Bobwhite' via biolistics. Eleven transgenic plants were recovered, and the response to head and leaf infection was evaluated in the progeny (T_1 and T_2 plants). The MoT isolate T-25, which carries a functional allele of *AVR-Piz-t*, was used for inoculations. Disease severity was scored as percentage of affected spikelets or percentage of leaf area affected, in head and leaf assays, respectively. Two transgenic lines, Piz-t_5238.C.1 and Piz-t_5503.C1 showed a slight reduction in susceptibility to wheat spike blast (WsB), but seedlings of these same lines showed significant reductions in the percentage of leaf area affected, suggesting that *Piz-t* could confer some resistance to wheat leaf blast (W_LB). Wheat transgenic lines expressing

antimicrobial peptides (AMPs) were also challenged with the wheat blast pathogen. Transgenic wheat plants were expressing, independently, the peptides Ace-AMP1, WD, ARACIN1, and Zeamatin. Fourteen AMP lines were tested in head assays, and 7 lines were tested in leaf assays. Significant reductions in the severity of wheat blast were not observed, indicating that the AMPs evaluated in this study did not confer resistance to either W_SB or W_LB. The introduction of rice blast resistance genes into wheat might be a valuable alternative to traditional breeding to incorporate resistance to wheat blast. Because the *AVR-Pi9* and *AVR-Pi54* genes are widely distributed in MoT populations, the resistance genes *Pi9* and *Pi54* would be good candidates for future studies.

Introduction

Wheat blast disease, caused by the fungus *Magnaporthe oryzae* (Hebert) Barr (synonymous with *Pyricularia oryzae*) (Couch & Kohn, 2002), is a very limiting disease for wheat production in South America and Bangladesh, and a serious threat for global wheat production. The disease is caused by a specific lineage of *M. oryzae*, the *Triticum* pathotype (MoT), and this lineage is distinct from others pathotypes infecting other plant species such as the *Oryza* pathotype (MoO), *Lolium* pathotype (MoL), *Eleusine* pathotype (MoE), or *Setaria* pathotype (MoS), which infect rice, turf and forage grasses, finger millet, or foxtail millet, respectively (Gladieux et al., 2018; Valent et al., 2019).

Wheat blast was first reported in the Paraná State of Brazil in 1985 (Igarashi et al., 1986), subsequently it was reported in Bolivia in 1996 (Barea & Toledo, 1996), and it quickly spread to Paraguay (Viedma & Morel, 2002) and Northern Argentina (Cabrera & Gutierrez, 2007; Perelló et al., 2015). The disease was confined to South America for more than 30 years, but in 2016 it was first reported in Bangladesh (Malaker et al., 2016). Analysis of the Bangladeshi isolates

showed a high genetic identity with South American isolates (Malaker et al., 2016; Islam et al., 2016), and it is believed that the disease may have entered Bangladesh through contaminated seed from Brazil (Ceresini et al., 2019). Unofficial reports indicated that the disease was found affecting wheat fields in two districts of India, on the border with Bangladesh (Bhattacharya & Pal, 2017).

Disease development is favored by high temperatures (18 to 30°C), high relative humidity, frequent rain periods, and a minimum of 25 hours of spike wetness (Cardoso et al., 2008; Kohli et al., 2011). Under favorable conditions, losses due to this disease can reach up to 100%, as reported for epidemics in South America and Bangladesh (Duveiller et al., 2016; Islam et al., 2016). The main sources of inoculum are crop residues and secondary hosts, from where the conidia become airborne (Kohli et al. 2011). Studies have suggested that conidia produced in wheat leaves may be a potential source of inoculum (Cruz et al., 2015; Cruppe, 2020a). In addition, MoT is also seed-transmitted, and it can infect the seedling as it emerges (Cruz & Valent, 2017). Although MoT can infect all the aerial parts of the plant (Igarashi et al., 1986), the most typical symptom of the disease is the whitening or bleaching of the heads, which results from a blockage in the translocation of nutrients due to infection of the rachis (Cruz & Valent, 2017; Duveiller et al., 2016). Wheat blast reduces yield and seed quality, and major impacts occur when infection take place during anthesis or early grain development, because seed produced by infected heads are shriveled, deformed, small and light weight (Goulart et al., 2007; Duveiller et al., 2016). If the infection occurs late, the seeds develop better, but this can increase the risks of seed transmission (Cruz & Valent, 2017).

The management of the disease has been a challenge as cultural practices and chemical control have not been very efficient, especially when susceptible genetic backgrounds are used

(Islam et al., 2019; Kohli et al., 2011). In addition, the development of resistance to fungicides has also been reported (Castroguadin et al., 2015). The use of resistant wheat cultivars may be the best strategy to control the disease, however commercial cultivars with proven resistance are not available (Islam et al., 2019). Although nine resistance (*R*) genes have been identified in common wheat and in tetraploid wheat, *Rmg1* to *Rmg8* and *RmgGR119* (Anh et al., 2015; Nga et al., 2009, Tagle et al., 2015; Takabayashi et al., 2002; Vy et al., 2014; Wang et al., 2018; Zhan et al., 2008), not all of these genes confer resistance to the *Triticum* pathotype. For example, *Rmg1* (*Rwt4*) prevents infection of the *Avena* isolates carrying the corresponding avirulence (*AVR*) genes *PWT3* and *PWT4* (Inoue et al., 2017; Takabayashi et al. 2002), whereas *Rmg4*, *Rmg5* and *Rmg6* confer resistance to *Digitaria* and *Lolium* isolates (Nga et al., 2009; Vy et al., 2014). The remaining five genes, *Rmg2*, *Rmg3*, *Rmg7*, *Rmg8* and *RmgGR199*, were shown to be effective against MoT in laboratory studies (Anh et al., 2015; Tagle et al., 2015; Wang et al., 2018; Zhan et al. 2008); however, some of them are temperature sensitive while others are not highly effective against the new, and apparently more aggressive, MoT isolates (Cruz & Valent, 2017). Few *AVR* genes, few of these genes have been identified in MoT. Besides the *PWT3* and *PWT4* genes mentioned previously (Inoue et al., 2017), the *AVR-Rmg8*, recognized by the resistance genes *Rmg8* and *Rmg7*, was recently isolated (Anh et al., 2018). In addition to the *R* genes described, Cruz et al. (2016a) reported head blast resistance in some wheat materials and near-isogenic lines (NILs) carrying the 2NS/AS chromosomal translocation from the wild wheat *Aegilops ventricosa*. However, not all lines carrying the translocation showed a significant reduction, and the response seems to be dependent on the MoT isolate used. The gene or genes responsible for the resistance to MoT in the 2NS fragment have not been cloned, but this fragment was already incorporated into several cultivated wheat varieties because it carries

resistance genes to other wheat diseases (Cruz & Valent, 2017). The emergence of more aggressive isolates has led to a search for new materials that offer new sources of resistance. In a recent study, Cruppe et al. (2020b) found only 8 resistant or moderately resistant accessions out of more than 780 accessions evaluated, including four non-2NS breeding lines from CIMMYT (International Maize and Wheat Improvement Center) and four accessions of the wheat wild-relatives *Aegilops tauschii*. The limited availability of materials with resistance to MoT and the limited knowledge about the gene(s) associated with the resistance to MoT in the main source of resistance, the 2NS translocation, makes the implementation of new approaches a priority.

Few studies aimed to understand the interaction between wheat and MoT have been developed, perhaps because the disease was reported recently. Conversely, the rice blast disease caused by *M. oryzae Oryza* pathotype (MoO) has been extensively studied. Rice blast is an ancient disease, and in the arms race between MoO and rice, several blast resistant (*R*) genes and avirulence (*AVR*) genes have coevolved. More than 100 *R* genes have been identified in rice, of which 35 have been cloned, whereas 12 *AVR* genes have been cloned in MoO (reviewed by Wang et al., 2017; Kalia & Rathour, 2019). In addition to the characteristic *AVR* genes recognized by *R* genes, other genes that contribute to the biotrophic invasion of MoO (*BAS* genes) have been described (Mosquera et al., 2009). The introduction of rice *R* genes into the wheat genome may be an alternative strategy to improve resistance to wheat blast. However for this strategy to be effective, the corresponding *AVR* genes must be present in the MoT population. In a recent study, Peng et al. (2019) sequenced the genome of six MoT isolates, and isolates from other hosts, and they found that several homologs to MoO-effector genes were present in these isolates, particularly, nine genes (*AVRPiz-t*, *AVR-Pita3*, *AVR-Pib*, *AVR-Pi54*, *AVR-Pi9*, PWL4, BAS2, BAS3 and BAS4) were found in the genome of the 6 MoT isolates

analyzed (Peng et al., 2019). This was the first report describing the presence of genes homologous to MoO-effectors in MoT, but few isolates were evaluated. One of the objectives of this study was to characterize the presence/absence of genes homologous to the MoO-effector genes in a group of MoT isolates collected from infected wheat between 1986 and 2017, in different producing regions in Brazil, Bolivia and Paraguay. The second objective was to select a rice resistance gene, based on the frequency of occurrence of the corresponding *AVR* gene in the MoT population, and introduce it into a susceptible wheat material to evaluate the potential utility of this transgenic approach to generate resistance to MoT.

As an alternative to *R* genes, enhanced resistance to MoO has been obtained by the introduction of genes from unrelated organisms into rice by genetic transformation, and promising results have been achieved by the expression of genes encoding antimicrobial peptides (AMPs). AMPs are small peptides that are part of the innate immune response of most living organisms against potential pathogens, and whose mode of action is associated with membrane damage, interaction with intracellular targets or modulation of the host defense response (Kang et al., 2017). AMPs from fungi, insects and plants expressed in rice plants showed growth inhibition *in vitro* and *in vivo*. For example, the expression of an antifungal protein AFP from *Aspergillus giganteus* in transgenic rice resulted in a strong inhibition of MoO growth, due to an abnormal development of the hyphae on leaves from transgenic plants (Coca et al., 2004). Similar results were observed when the *cecropin A* gene from the moth *Hyalophora cecropia*, was transformed into rice (Coca et al., 2006). Thanatin, from the stinkbug *Podisus maculiventris*, also conferred enhanced resistance to rice blast, reducing the disease progress by up to 50% (Imamura et al., 2010). Plant AMPs, like wheat puroindolines A and B, the nonspecific-lipid transfer Ace-AMP1 from *Allium cepa*, and the defensins Dm-AMP1 from *Dahlia merckii*, Mj-

AMP2 from *Mirabilis jalapa*, and WD from wasabia (*Eutrema japonicum*), were also expressed in transgenic rice plants and significant reductions in rice blast symptoms were reported for all of them (Jha et al., 2009; Kanzaki et al., 2002; Krishnamurthy et al., 2001; Patkar & Chattoo, 2005; Prasad et al., 2008). Since the potential activity of plant AMPs against MoT has not been tested, genes encoding the peptides Ace-AMP1, WD, ARACIN1, and Zeamatin, were chosen for this study. The description of these four peptides was previously presented in Chapter 2. The third objective of this study was to evaluate the response of transgenic wheat plants, expressing independently each peptide, to MoT infection.

Materials and Methods

M. oryzae isolates and DNA extraction

M. oryzae isolates used in this study are maintained in the Wheat Blast laboratory (Biosafety Level 3 – BSL3) at the Biosecurity Research Institute (BRI), Kansas State University, Manhattan. The current collection include isolates from Brazil, Bolivia and Paraguay, collected between 1986 to 2017, from wheat and other grasses. The isolates are preserved on filter paper and kept at -20°C or -80°C (long term). A representative group of 127 isolates was selected, including isolates collected in different years and wheat growing regions. One hundred twenty-four of the selected isolates were obtained from wheat samples, two from *Lolium multiflorum* and one from *Digitaria horizontalis*. If the paper-preserved isolates were previously purified by single spore isolation, they were used directly to produce mycelium. If they were not, they were purified in this study, preserved in filter paper and included in the collection. To recover the isolates, pieces of filter paper with fungus growth were placed in petri dishes with oatmeal agar (Valent et al., 1991), and plates were incubated for 6 to 8 days at 25°C under constant light (fluorescent lighting), until sporulation was observed. Twenty-one of the 127 isolates were not

recovered, and the following steps were performed with the remaining 106 isolates (information related to these isolates is presented in the Table 3.1). Sporulated colonies were covered with approximately 5 mL of liquid 3-3-3-medium (0.3% w/v glucose, 0.3% w/v yeast extract, 0.3% w/v casamino acids), the surface of the colony was gently scraped with a spatula to release the conidia, and the conidial suspension was transferred to a 250 mL Erlenmeyer flask containing 150 mL of liquid 3-3-3 medium. Flasks were incubated in the dark for 3 days at 25°C with constant shaking (150 rpm). After incubation, the mycelium was harvested by filtration through 6 layers of sterile cheesecloth, washed with sterile distilled water, and the excess liquid was removed by squeezing the mycelial pellet with sterile paper towels. The mycelium was conserved in aluminum foil envelopes at -80°C. DNA was isolated using the Quick-DNA™ Fungal/Bacterial Miniprep kit (Zymo Research, Irvine, CA), following the manufacture's protocol, and homogenizing the cells with a Bead Ruptor 4 (OMNI International, Kennesaw, GA) at maximum speed for 6 minutes. DNA samples were stored at -80°C until they were transferred to a BSL1 laboratory. Before moving the samples from the BSL3 laboratory, a risk assessment protocol was completed to confirm the inactivation of *M. oryzae* in the DNA samples. The samples were transferred to a BSL1 laboratory after the report was approved by the BRI biosafety staff. DNA quality was analyzed by electrophoresis in 1% agarose gels in 1X TAE buffer and quantified by NanoDrop (ThermoScientific, Waltham, MA).

Amplification of homologs of MoO-effector genes in MoT isolates

The presence/absence of genes homologous to the MoO-effector genes in MoT isolates was evaluated by conventional PCR. The primers used to amplify the effector genes were previously reported (Shi et al., 2018; Peng et al., 2019) or designed in this study, using the MoO sequences available in the GeneBank. All primers were synthesized by IDT (Integrated DNA

Technologies, Coralville, Iowa) and sequences are shown in Table 3.2. In addition, Pot2 primers, which amplify a fragment of the Pot2 transposon present in isolates of *M. oryzae* from various hosts, and MoT3 primers, which specifically amplify *M. oryzae* isolates from *Triticum* (Pieck et al., 2017) were used as positive controls. The presence/absence of the *AVR-Rmg8* gene, recently identified in a MoT isolate (Ahn et al., 2018), was evaluated using the primers reported by Wang et al. (2018). PCR was performed in a 25 µL reaction containing 1X GoTaq® Flexi buffer (Promega, Madison, WI), 2.5 mM $MgCl_2$, 400 µM each dNTP, 0.4 µM each primer, 1 unit of GoTaq® Flexi DNA polymerase (Promega, Madison, WI), and 25 ng genomic DNA. All reactions were completed on a Multigene Gradient thermal cycler (TC9600G, Labnet, Edison, NJ) with initial denaturation at 94°C for 5 min, followed by 35 cycles of 94°C for 30 seconds, corresponding annealing temperature for 30 sec, and 72°C for 45 sec, and a final extension at 72°C for 10 min. An annealing temperature of 56°C was used with the primers PWL2 a/b, Avr-Pii 3/4, Avr-pita1 g/h, Avr-pita3 c/d, Avr-Rmg8 F1/R1; 58°C were used with Pot2a L2/R2, Avr-Pia 5/6, Avr-Pik a/b, SCO12 P1/P2, Avr-Pi54 F2/R2, BAS4 F1/R1; 59°C were used with PWL1 F1/R1, PWL3 F1/R1, PWL4 F1/R1, Avr-Pib F1/R1, Avr-Pi9 F1/R1, BAS1 F/R, BAS2 F1/R1, BAS3 F1/R1; 60°C were used with MoT3 F/R, Avr-pita2 1/2, Avr-Piz-t a/b; and 61°C were used with ACE1 23/10 and Avr1-CO39 12/13. PCR products were analyzed by electrophoresis in 1.0% agarose gels in 1X TAE buffer. PCR products obtained with the primers SCO12 P1/P2, Avr1-CO39 12/13 and Avr-pita3 c/d from some samples were purified with the QIAquick® PCR purification kit (QIAGEN, Germantown, MD) and sent to sequencing to Genewiz (South Plainfield, NJ).

DNA plasmids for wheat transformation

The rice resistance gene *Piz-t* encodes a nucleotide-binding site leucine-rich repeat (NBS-LRR) protein of 1033 amino-acids (Zhou et al., 2006). The plasmid pPiz-t_C1305 (21088 bp), donated by Dr. Guo-Liang Wang (Ohio State University), was constructed by cloning the *Piz-t* gene with its endogenous rice promoter into the pCAMBIA C1305 vector, with the NOS terminator. The plasmids Ace1_pAHC17, Wj1_pAHC17, ARA1_pAHC17, and Zma_pAHC17 containing the AMP genes under the control of the maize *Ubiquitin1* (*Ubi-1*) promoter and *Nopaline Synthase* terminator (*tNOS*) were described in detail in Chapter 2. The plasmid pAHC20 with the *bar* gene (confers resistance to the herbicide glufosinate) under the control of the *Ubi1* promoter (Christensen & Quail, 1996) was used in co-transformation experiments to select plant material during tissue culture process and putative transgenic plants.

Biolistic transformation of wheat and identification of positive transgenic plants

Transformation experiments were done using embryogenic calli of the spring wheat cultivar Bobwhite (BW; CM33203; released in 1984 by CIMMYT), according to the methodology described by Tian et al. (2019). In Chapter 2, the protocols for transforming wheat calli with the AMP genes by biolistic, regeneration of plantlets, and identification of positive transgenic plants by herbicide "painting" and conventional PCR were described in detail. Transgenic wheat lines expressing the AMP genes described in Chapter 2 were the same lines challenged with MoT in this study.

The standardized protocols for tissue culture, plant transformation and plant recovery described in Chapter 2 were used to generate *Piz-t* transgenic plants. In co-transformation experiments, tungsten particles were coated with the DNA plasmids pPiz-t_C1305 and pAHC20, using 2 μL of each one. Regenerated plantlets were evaluated by herbicide "painting", and the

presence of the transgenes *Piz-t* and *bar* was verified in putative transgenic plants by PCR, using specific primers designed to amplify each transgene. In addition, tubulin primers were used as control (Table 3.3). PCR was done in a 25 μL reaction containing 1X GoTaq® Flexi buffer, 2.5 mM $MgCl_2$, 400 μM each dNTP, 0.4 μM each primer, 1 unit of GoTaq® Flexi DNA polymerase (Promega, Madison, WI), and 25 ng genomic DNA in a final volume of 25 μL. Reactions were completed on a Multigene Gradient thermal cycler with 95°C for 3 min, 35 cycles of 95 °C for 30 seconds, 58°C for 30 sec, and 72°C for 45 sec, with a final extension at 72 °C for 10 min. PCR products were analyzed in 1.0% agarose gels in 1X TAE electrophoresis buffer.

Seeds from T_0 transgenic plants were harvested by collecting each tiller individually. Plants/seeds were labeled using the transgene name (Piz-t), followed by the number assigned to the shoot (consecutive number assigned in the Plant Transformation Lab) and followed by a letter representing the tiller. Seeds from individual plants in following generations (T_1, T_2, T_3, etc) were harvested together (bulk). Numbers added after the tiller designation represent the particular identification of each individual plant in the next generation (T_1, T_2, T_3, etc).

Expression of Piz-t in positive T_0 plants, relative expression and determination of the transgene copy number

Expression of *Piz-t* was evaluated in positive T_0 plants/tillers and in plants from the next generations that were used in bioassays. Tissue was collected (about 100 mg of leaf tissue, ≈ 8 cm foliar lamina) in 2.0 microcentrifuge tubes containing two 4.5 mm beads (BBs, Daisy®, Rogers, AR) and flash frozen in liquid nitrogen. Tissue was ground in a TissueLyser (QIAGEN, Germantown, MD), alternating homogenization for 25 sec at 25 Hz and freezing in liquid nitrogen, for a total of 3 homogenizations. Total RNA was isolated using 1 mL TRIzol® reagent (Invitrogen, Carlsban, CA), following the manufacture's protocol. The RNA pellet was

resuspended in 100 μL of RNase-free water, quantified by NanoDrop (ThermoScientific, Waltham, MA) and stored at -80°C. Single-stranded cDNA was synthesized by reverse transcription from 1 μg of total RNA using the Reverse Transcription system with Oligo$(dT)_{15}$ Primer (Promega, Madison, WI). The reverse transcription reaction was done in accordance with manufacturer's instructions, incubating the reaction at 42°C for at least 1 h. After the reverse transcription, 4 μL of cDNA were used as template in conventional PCR (Reverse Transcription-PCR or RT-PCR). Amplification was completed following the conditions previously reported to identify transgenic plants, using the specific primers for *Piz-t* and *bar*. *Tubulin* primers (Tub-F and Tub-R) were used to test contamination with genomic DNA (gDNA), since the PCR products obtained from gDNA are 500 base pairs (bp), while the products obtained from cDNA are 408 bp. Expression of the *Piz-t* gene was also evaluated in heads. For this, total RNA was isolated using the RNeasy mini kit (QIAGEN, Germantown, MD), according to manufacturer's recommendations. cDNA synthesis and RT-PCR were conducted following the same procedures described for leaves.

A two-step quantitative Real-Time PCR (Reverse Transcription-qPCR or RT-qPCR) assay was used to determine the relative expression of *Piz-t* in T_0 plants. Primers were designed based on the sequences available in the GeneBank, accessions Q5EWZ1 and DQ352040 for the reference gene *actin* and *Piz-t*, respectively (Table 3.3). The optimum annealing temperature (T_a) was established through a thermal gradient assay with a range of temperatures: 56.6, 58.2, 60.1, and 61.7 °C. The qPCR conditions used were: 1X SsoAdvanced universal SYBR® Green supermix (Bio-Rad, Hercules, CA), 0.35 μM each primer, and 5 μL diluted cDNA (1:4 with RNase-free water), in a 20 μL final volume. The reactions were completed using the Bio-Rad CFX96 Real-Time System (Bio-Rad, Hercules, CA) with polymerase activation/denaturation at

95°C for 30 sec, 40 cycles with denaturation at 95°C for 15 sec and annealing/extension at each temperature for 30 sec, and a melt curve analysis with 65°C to 95°C, with 0.5°C increments each 5 sec. Each sample was run in triplicate, and data were collected using the CFX Maestro™ Software (Bio-Rad, Hercules, CA). A standard curve using the optimized Ta was run to test the efficiency of primers. Serial 10-fold dilutions were prepared using a positive control DNA, and each dilution was run in triplicate using the conditions reported before for the gradient assay. Primers with percentages of efficacy between 90 and 110% and coefficients of determination (R^2) > 0.98 for the standard curve were used in the following experiments. The relative expression of *Piz-t* in the T_0 transgenic plants was determined using the standardized RT-qPCR conditions. Three technical replicates were run for each sample, and the relative expression was calculated considering the expression level of the reference gene (*actin*) and the expression level of the target gene (*Piz-t*), according to the formula: Expression = $2^{\Delta Ct} = 2^{(Ct\ actin) - (Ct\ target)}$.

The transgene copy number was estimated using droplet digital PCR (ddPCR). The wheat *Puroindoline-b* (*Pinb*) gene present as two copies in the genome (5D chromosome) was used as reference gene. Primers to amplify the *Pinb* gene (accession DQ363914) were published by Collier et al. (2017), and primers for *Piz-t* were designed using the sequence reported in GeneBank accession DQ352040 (Table 3.3). The optimal annealing temperature (T_a) was determined by a gradient PCR, with a range of annealing temperatures from 54°C to 62°C. ddPCRs were completed in 20 µl reactions with 1X QX™ ddPCR™ EvaGreen supermix (Bio-Rad, Hercules, CA), 140 nM each primer, 3 units *Hind*III-HF (New England BioLabs, Ipswich, MA), and 37.5 ng DNA, and they were stored overnight at 4°C. Before generating the droplets, the reactions were incubated at room temperature. The droplets were generated in a QX200 Droplet Generator (Bio-Rad, Hercules, CA), in accordance with the manufacturer's instructions.

Droplets (40 μL) were carefully transferred to a 96 well plate, and amplification was completed in a C1000 Touch thermal cycler (Bio-Rad, Hercules, CA) with enzyme activation at 95°C for 5 min, 40 cycles with denaturation at 95°C for 30 sec and annealing/extension for 1 min, signal stabilization at 4°C for 5 min and 90°C for 5 min; with a ramp rate of 2°C/sec. The annealing temperatures for the gradient were 54, 54.6, 55.7, 59, 60.5, 61.5 and 62°C. After the amplification, the plate was read in the QX200 Droplet Reader, and the data was analyzed with the QuantaSoft™ Software. The optimum annealing temperature was that in which the greatest number of positive droplets was generated, and the separation of positive and negative droplets was clearly defined. To measure the transgene copy number in the T_0 plants, the samples were amplified following the methodology described above, using the optimum Ta determined in the gradient assay. Samples were evaluated with the two sets of primers (dd_Piz-t_F/R and ddPinb_F1/R1) in independent reactions (wells). The concentration of each gene (copies/μL) was estimated with the QuantaSoft™ Software, and the transgene copy number was calculated according to the formula: [transgene concentration (copies/μL) / reference concentration (copies/μL)] x 2.

Bioassays

Although *M. oryzae Triticum* pathotype infects all the aerial parts of wheat, the head infection is the most common symptom and has the greatest impact. Therefore, evaluations of the response of the transgenic plants to MoT focused on head infection (wheat spike blast – W_SB). However, leaf infection may have a role in the production of inoculum in the field, thus some experiments were conducted to test the response of transgenic lines to the leaf infection (wheat leaf blast – W_LB).

Transgenic plants from different generations (T_1, T_2, T_3 or T_4) and controls (Bobwhite_wild type and a transgenic line carrying and expressing the *bar* gene - bar_8210.C.4) were grown in chambers with 18°C/15°C day/night temperature, a 16 h photoperiod, a light intensity of 450 μE m^{-2} s^{-1}, and 50 to 60% relative humidity, in the facilities of the Throckmorton Plant Sciences Center. For leaf inoculation, plants were grown to the two- or three- leaf stage (Feekes growth 1.0, before tillering), while for head inoculations, plants were grown until they reached the boot stage (Feekes growth scale 10.0). During vegetative growth, transgenic plants were tested for the presence and expression of the transgenes. Direct amplification with the KAPA3G Plant PCR kit (Kapa Biosystems, Indianapolis, IN) was used to test the presence of the transgenes. Briefly, a piece of leaf lamina (approximately 0.5 cm x 0.5 cm) was cut with scissors and transferred to a microcentrifuge tube (1.5 ml) containing 100 μL of extraction buffer (50 mM Tris-HCl pH 8.0, 0.1 mM EDTA pH 8.0, 2% β-mercapto-ethanol). The tissue was crushed with a micropipette tip and preserved on ice. Tubes were incubated at 95°C for 5 minutes and put back on ice. The crude extract was diluted at 1:10 with double-distilled water (ddH_2O) and used freshly in PCRs. The PCR conditions were 1X KAPA PCR buffer, 1 mM $MgCl_2$, 0.3 μM each primer, 1 unit KAPA3G polymerase, 5 μL of diluted crude extract, in a final volume of 25 μL. PCRs were completed in a Multigene Gradient thermal cycler (TC9600G, Labnet, Edison, NJ) with 95°C for 6 minutes, 40 cycles of 95°C for 30 seconds, 58°C for 30 seconds, and 72°C for 45 seconds, and a final extension at 72°C for 10 minutes. The expression of the transgenes was evaluated in positive transgenic plants by RT-PCR, following the methodology described above.

When the plants reached the appropriate development stage, they were transported to the Biosecurity Research Institute and introduced to the Wheat Blast Laboratory (Biosafety Level 3 – BSL3). All the inoculations were conducted using the *M. oryzae Triticum* pathotype isolate T-

25 (BR88Ta025), collected in Sao Jorge Do Ivar, Brazil in 1988. Isolate T-25 was used in this study because it has the *AVR-Piz-t* functional allele (M. Farman, University of Kentucky, personal communication). Specifically, the MoT allele was transformed into a rice pathogen and the transformant was avirulent on rice with the *Piz-t* resistance gene. The production of inoculum and inoculation was previously standardized and reported by Cruz et al. (2012; 2016b). Briefly, pieces of filter paper with fungus growth were placed in oatmeal agar (Valent et al., 1991) and the plates were incubated at 25°C, under light, for 6 to 8 days. To prepare the conidial suspension (inoculum), approximately 8 mL of gelatin-Tween20 solution (0.42% w/v gelatin, 0.01% v/v Tween20) were added to the petri dish and the surface of the colony was gently scraped off with an inoculation ring to release the conidia. The suspension was filtered through four layers of sterile cheesecloth, and the concentration was adjusted to 20,000 conidia per mL using a disposable hemocytometer (C-Chip, INCYTO, SKC Inc., Covington, GA). Heads were inoculated 1 or 2 days after full head emergence (Feekes growth scale 10.5 to 10.5.1), by spraying approximately 0.75 mL of the conidial suspension using an airbrush (Harbor Freight Tools, Camarillo, CA). Inoculated heads were immediately covered with black resealable plastic bags (7.6 x 12.7 cm, Ref. S-12322BL, ULINE, Coppell, TX) previously moistened with water inside, and they were removed after 48 h. Inoculations were done every other day, using freshly prepared inoculum and selecting the plants that were in the appropriate developmental stage, until all the plants were inoculated. For the inoculation of leaves, seedlings growing in cells in 10 x 20 trays (Ray Leach single cell cone-tainer system, Hummert, Earth City, MO) were sprayed with 60 mL of the conidial suspension (for 100 seedlings - aprox. 0.6 mL per seedling). The tray was placed in a homemade wet chamber (a structure of PVC pipes, covered with a black plastic bag, with the inner walls moistened with water) and removed after 48 hours. The volume of

conidial suspension was adjusted to the number of plants evaluated. Adult plants and trays were kept in a growth chamber with 24°C/20°C day/night temperature, a 14 h photoperiod, with at least 300 μE m^{-2} s^{-1} light intensity, and 60 to 70% relative humidity. Disease severity was scored as percentage of affected area for leaf inoculations and percentage of affected spikelets for head inoculations. When the response to the disease was evaluated at several time points (days after inoculation - dai), the area under disease progress curve (AUDPC) was calculated using the trapezoidal method proposed by Madden et al. (2007). Analysis of variance (ANOVA) of the variables (AUDPC or an specific evaluation point) were done using the GLM procedure in the SAS software, Version 3.8, Enterprise Edition. Copyright© 2012-2018 SAS Institute Inc (Cary, NC).

Seven independent head inoculation experiments were were completed. Specific conditions of each of them were: ***Bioassay 1*** and ***Biosassay 2****:* four plants per transgenic line were used, and 3 heads per plant were inoculated. The disease severity (% of affected spikelets) was scored 10 days after inoculation (dai). ***Bioassay 3*** and ***Bioassay 4***: twelve plants per transgenic line were used, inoculations were done only on the first head (main tiller) of each plant. Disease severity was rated every other day, from 6 to 12 dai. ***Bioassays 5*** to ***7***: twelve plants per transgenic line were used, the main head of each plant was inoculated. Disease severity was scored every other day, from 6 to 14 dai.

Leaf response to MoT infection was evaluated in four independent experiments. ***Bioassay 8*** and ***Bioassay 9***: ten seedling per transgenic line were used, and the disease severity (percentage of leaf area affected) was scored 8 dai. ***Bioassay 10*** and ***Bioassay 11***: eight seedlings per transgenic line were evaluated, and the disease severity was rated every other day from 4 to 12 dai.

Results

Characterization of effector genes in MoT

A total of 106 isolates were evaluated with 24 pairs of primers. A band of the expected size (389 bp) was amplified in all isolates using the Pot2a primers (Figure 3.1). Seventy-eight isolates amplified positive bands with MoT3 specific primers, 77 of these isolates were obtained from wheat, but the isolate obtained from *D. horizontalis* also amplified. Twenty-eight isolates did not amplify with the MoT3 primers, including the two isolates of *L. multiflorum* and 26 isolates obtained from wheat, mainly Brazilian isolates collected in 2016 (Table 3.4 and Table 3.5). PCR amplicons with the expected size were obtained from almost all the pairs of primers used, with some exceptions. In 18 MoT isolates, the amplicon obtained with the primers Avr-pita3-c/Avr-pita3-d showed the expected size, but in 69 isolates, the amplicon was larger. Sequence analyzes showed that the heaviest amplicons have an insertion of 74 base pairs, when compared to the amplicons of the other isolates and the sequence reported in the GeneBank (accession DQ855957), and it has no homology with a known sequence. Amplification with primers Avr1-CO39-12/Avr1-CO39-13 resulted in PCR products with the expected size in 79 isolates, but in 11 isolates, the PCR band was weak (faint) and slightly short. Sequences from these PCR products showed multiple peaks (high background), while the sequences of the expected amplicons were similar to the sequenced reported in the GenBank (accession AF463528). PCR amplicons obtained with the primers SCO12-P1 and SCO12_P2 (AVR-Pik^{m}) showed the expected size, but the bands were faint, and the sequence of the samples tested showed multiple peaks, which could indicate the possible presence of different alleles, as observed in *AVR-Pik* (Kanzaki et al., 2012). The results of the amplifications with the 24 pairs of primers, in the 106 Mo isolates are presented in the Table 3.4. The isolates from *Lolium* and

Digitaria, and the isolate KY-5506 collected in Kentucky in 2011, were not included in the frequency analysis.

The frequency of occurrence of the 22 effector genes evaluated (21 homologs of the MoO effector genes and the MoT *AVR-Rmg8*) is presented in Figure 3.1. Three genes, *PWL1*, *AVR-Pik*, *AVR-pita1*, were not amplified in any of the MoT isolates evaluated. Three genes, *AVR-Pia*, *AVR-Pii*, and *AVR-pita2*, were only amplified in 1, 3 and 1 isolate(s), respectively. The A*VR* genes *AVR-Piz-t, AVR-Pi9*, *AVR-Pi54,* and *ACE1*, and the genes encoding for biotrophy-associated secreted proteins *BAS2*, *BAS3* and *BAS4*, occurred at the highest frequency. In general, most effector genes were distributed in isolates from the three countries evaluated Brazil, Bolivia and Paraguay, although *PWL3* only amplified in 17 Brazilian isolates, while *AVR-Pia* and *AVR-pita2* only amplified in a Bolivian isolate collected in 2014 (Table 3.4 and Table 3.5). Although a representative number of isolates from the three countries in all years of collection was not available, and a limited number of isolates was available for some years, a trend in the frequency of occurrence of PWL2 and BAS1 is observed, which has been increasing over the years (Table 3.5).

A group of 19 Brazilian isolates, including two MoL and 17 MoT, collected in 2016 in Santa Rosa and Coxilha, showed a different amplification profile from that of most Brazilian isolates. For example, none of these isolates was positive for the MoT3 marker, nor for the *AVR-Pib* gene, 17 of the 19 isolates were negative for *PWL4*, but 18 of them were positive for *PWL3* (Table 3.4). Likewise, three Bolivian isolates collected in Santa Cruz (Okinawa2) in 2014, did not amplify many of the genes commonly found in the other isolates evaluated, such as *PWL4*, *AVR-Piz-t*, *AVR-Pi9*, *ACE1*, *AVR-Pib*, *BAS1* and *BAS2*.

Recovery and molecular characterization of *Piz-t* transgenic plants

Fourteen independent biolistic transformation experiments were performed with pPiz-t_C1305, using a total of 1710 embryogenic calli. One hundred and seventy five plants were tested by "painting", and 37 plants showed resistance to the herbicide. Molecular analyzes showed that transgenes *Piz-t* and *bar* were present together in 11 plants, but in 22 plants only *bar* was present. Overall, the transformation efficiency with *Piz-t* was 0.64%.

Expression analysis (RT-PCR) of 10 T_0 transgenic plants showed co-expression of *Piz-t* and *bar* genes. Expression in heads was also positive for all the plants evaluated. High variation in the relative expression of *Piz-t* among T_0 plants was observed (Figure 3.2). The plant Piz-t_4739 was not evaluated either by RT-PCR or by RT-qPCR because no RNA was obtained from it. Measurements of the copy number estimated by ddPCR were close to integer values in 9 samples, but the copy number was estimated to be mid-way between integers (i.e. between .4 and .6) in 2 samples: Piz-t_5503.C and Piz-t_8212.B. T_1 seeds were recovered from all T_0 plants, and lines were advanced until T_3 generation. Segregation of the transgene (progeny without the *Piz-t* gene) was observed in some lines. T_1 plants from the line Piz-t_8210 were not carrying *Piz-t*, but they were carrying and expressing *bar*, and this gene was inherited in the next generations up to it was evaluated (T_3). This line was re-named as *bar*_8210 and it was used as control in the bioassays. Silencing of *Piz-t* was observed in T_1 plants that came from Piz-t_4739.A (tiller A), whereas T_1 plants derived from the tiller C (Piz-t_4739.C) were expressing the transgene. However, the gene was silenced in the T_2 generation. Silencing was also observed in T_1 plants derived from Piz-t_8212.B, but T_1 plants from Piz-t_8212.A were expressing the gene, and the expression was confirmed in the next generation. Due to segregation and silencing of *Piz-t*, all plants used in the bioassays were tested for the presence and expression of the transgene.

Recovery and molecular characterization of wheat transgenic plants expressing AMPs were discussed in the Chapter 2. Some of the transgenic lines described in Chapter 2 were used in this study and challenged with MoT isolate T-25.

Bioassays

The response to spike infection (W_SB) was evaluated in seven independent bioassays, using 21 transgenic lines, 7 lines expressing the rice-resistance gene *Piz-t* and 14 lines expressing AMPs (3 Ace1 lines, 5 Wj1 lines, 3 ARA1 lines, and 3 Zma lines). The disease progress curves and the analysis of the AUDPC are presented in Figure 3.3 and Figure 3.5. The response to leaf infection (W_LB) was assessed in four independent bioassays, where 14 transgenic lines, 7 lines expressing the *Piz-t* gene, 3 expressing *WD*, 3 expressing *ARACIN1*, and 1 line expressing *Zeamatin* were tested. The response of these plants to W_LB is presented in Figure 3.4 and Figure 3.6.

In the ***Bioassays 1*** and ***2***, T_2 plants from 9 transgenic lines were tested, and the disease severity was only rated at 10 dai. Two lines, Wj1_8538.C.2 and Piz-t_5238.C.1 showed a statistically significant reduction in the percentage of spikelets affected, when comparted to the control and other lines. The line Piz-t_5503.C.1 also showed a reduction, although it was not significant (Figure 3.5 A and 3.5 B). T_2 plants from the lines Piz-t_5238.C.1 and Piz-t_5503.C.1 were tested again in the ***Bioassay 3***, and a reduction in the percentage of affected spikelets was observed in both lines across all evaluation days (6 to 12 dai) (Figure 3.3 A). This was reflected in a decrease in the AUDPC, although the difference was only significant for the line Piz-t_5238.C.1 (Figure 3.5 C). In the ***Bioassay 4***, three transgenic lines were tested, including T_3 plants from the line Wj1_8538.C.2, which showed a decrease in the percentage of affected spikelets in the *Bioassay 1*. A reduction in the percentage of affected spikelets was observed in

plants from lines Wj1_8538.C.2.15 and Wj1_8650.C.3 during the first few evaluation days (up to 8 dai), and this trend remained the same for line Wj1_8650.C.3 until 10 dai (Figure 3.3 B). AUDPC for both lines showed significant differences when compared to control and other transgenic lines (Figure 3.5 D). In the ***Bioassay 5***, four AMP lines were tested, and a slight reduction in the percentage of affected spikelets was observed in the line Ace1_8666.A.1 across the evaluation days (6 to 14 dai) (Figure 3.3 C), nevertheless, significant differences in the AUDPC were not detected (Figure 3.5 E). Seven transgenic lines were evaluated in the ***Bioassay 6***, and differences in the disease progress curves were observed in several lines, but even the control Bobwhite_WT showed a reduction in the percentage of affected spikelets (Figure 3.3 D). Statistical analyzes showed that the reductions in the AUDPC of the line Zma_8558.A.5.4 and Bobwhite_WT were significantly different (Figure 3.5 F). In ***Bioassay 7***, three of the lines previously evaluated were again challenged with MoT. T_3 plants of lines Piz-t_5238.C.1.66, Piz-t_5503.C.87, and Zma_8558.A.5.4 (evaluated in *bioassay 6*) were tested, and T_3 plants of line ARC1_8894.D.1.4 were included, which previously showed a reduction in the response to *Fusarium graminearum* infection (Chapter 2). The disease progress curves showed a decrease in the percentage of affected spikelets in the 4 transgenic lines (Figure 3.3 E), but significant differences in the AUDPC were only detected in the Piz-t_5503.C.87 and ARC1_8894.D.1.4 lines (Figure 3.5 G). Although plants from 5 transgenic lines showed a reduction in the percentage of spikelets affected (or in the AUDPC), and the differences were statistically significant, the percentages of affected area were above 82% (except for bioassay 6), indicating that transgenes incorporated in wheat did not confer resistance to W_SB.

The response of leaves to MoT infection was evaluated in 4 bioassays. In ***Bioassay 8*** and ***Bioassay 9***, T_1 plants from seven Piz-t lines and T_2 plants form five AMP lines were tested,

respectively. The disease severity was scored 8 dai as percentage of leaf area affected. Significant decreases in severity were not observed in any of the lines, however, a slight reduction in the average percentage of leaf affected was observed in lines Piz-t_5238.C, Piz-t_5503.C, and Piz-t_8212.B (Figure 3.6 A). Seedlings (T2) from lines Piz-t_5238.C.1 and Piz-t_5503.C.1 were evaluated again in the ***Bioassay 10***. A reduction in the percentage of affected area was observed in plants from line Piz-t_5503.C.1 across all evaluation days (4 to 12 dai), whereas the percentage of affected area in Piz-t_5238.C.1 was low during the first 8 days, but increased at the end of the evaluation (Figure 3.4 A). Significant differences were observed in the AUDPC from the line Piz-t_5503.C.1 (Figure 3.6 C). In the Bioassay 11, plants from three *WD* transgenic lines were evaluated. In the disease progress curve, a reduction in the percentage of affected area was observed in the line Wj1_8582.A.3 (Figure 3.4 B), and the AUDPC showed significant differences compared to other transgenic lines and controls tested (Figure 3.6 D). Overall, the results suggest that the expression of *Piz-t* in leaves may reduce susceptibility to W_LB.

Discussion

In the arms race between pathogens and plants, pathogens secrete molecules, called effector proteins, to manipulate the host and induce susceptibility, but plants produce resistance proteins that recognize these effectors and induce a strong resistance response (Sánchez-Vallet et al., 2018). Effectors play a crucial role in the interaction with the host, and the outcome depends on the host genotype. For example, resistance is triggered if the host recognizes the effector secreted by the pathogen, although effectors can promote infection in hosts that do not have the ability (resistance genes) to recognize them (Sánchez-Vallet et al., 2018). Effector genes and resistance genes in the *M. oryzae Triticum* pathotype – wheat interaction have been poorly

characterized. However, a large repertoire of effectors and resistance genes have been identified in the *M. oryzae Oryza* pathotype – rice interaction. In this study, the presence/absence in MoT isolates of genes homologous to MoO effectors was evaluated. The prevalence of these effector genes in MoT is the first step to identify rice resistance genes that can potentially be useful for controlling wheat blast. Here, 106 isolates (102 MoT, 3 MoL and 1 MoD) were tested using 21 pairs of primers to detect homologs to MoO effector genes, a pair of primers to detect the recently cloned MoT AVR-Rmg8 gene, and two pairs of primers to amplify Mo and MoT specifically. As expected, all the isolates amplified positive bands with the Pot2a primers, which were designed based on the sequence of the Pot2 transposon present in isolates of *M. oryzae* collected from different hosts (Pieck et al. 2017). In contrast, MoT3 primers designed to specifically amplify wheat blast isolates failed to detect 26 isolates from wheat (one of which, KY-5506, was a *Lolium* pathotype), but a positive band was detected in the isolate 17-MoD-40 from *D. horizontalis*. Pieck et al. (2017) reported that MoT3 diagnostic assay was useful to distinguish wheat isolates from a group of 284 *M. oryzae* isolates obtained from 11 different hosts, and they stated that the MoT3 marker was highly specific for the South American *Triticum* pathotype. In this study, the PCR reagents and thermocycling conditions used with MoT3 primers were not the same reported by Pieck et al. (2017). A debate about the use of this marker was raised when Gupta et al. (2019) reported that the MoT3 diagnostic assay could not distinguish between rice and wheat isolates from Bangladesh, although the authors admitted that the annealing temperature, and even the type of thermocycler used, influenced their results. A rebuttal paper highlighted several of the weaknesses of the experiments conducted by Gupta et al. (2019), including the lack of adherence to the explicit instructions reported by Pieck et al. (2017) (Yasuhara-Bell et al., 2019). The authors performed additional experiments and they

confirmed the reliability of the MoT3 assay to differentiate highly aggressive isolates in the *Triticum* lineage from isolates from other lineages (Yasuhara-Bell et al., 2019). Of the 26 isolates that did not amplify with MoT3, 18 of them are Brazilian isolates collected in 2016 in Santa Rosa and Coxilha, and this group, in particular, showed an effector amplification profile different from that of the other Brazilian isolates (Table 3.4). It is possible that Pieck et al. (2017) and Yasuhara-Bell et al. (2019) did not include such recent isolates in their studies, but Brazilian isolates from 2017 collected in different locations (Nao-me-Toque and Cascavel) were positive for MoT3, suggesting that the Santa Rosa-Coxilha population could be undergoing genetic changes.

Among the effector genes evaluated, three of them were not detected in any of the isolates, *PWL1*, *AVR-Pik*, *AVR-Pita1*. These results are supported by Peng et al. (2019), who in the sequence analysis of six wheat blast isolates did not find sequences homologous to these genes. Four out of six isolates included in Peng et. al (2019) study were also tested in this study, demonstrating consistency of results. The *AVR-Rmg8*, cloned from a wheat blast isolate (Anh et al., 2018) and recognized by the two wheat blast resistance genes *Rmg7* (Tagle et al., 2015) and *Rmg8* (Ahn et al., 2015), was detected in 85% of the MoT isolates tested. It was almost absent from the Bolivian isolates collected in 2014 (8 out of 9 isolates tested), from a Bolivian isolate collected in 2017, and in half of the Brazilian isolates collected in 2017 (Table 3.4). Interestingly, *AVR-Rmg8* was amplified in almost all Brazilian isolates collected in 2016 but was not detected in isolates 16-MoT-01 and 16-MoT-02. In a recent report, Cruppe et al. (2018) showed that 16-MoT-01 was very virulent to accessions carrying the 2NS fragment and to accessions without this resistance source. Since isolate 16-MoT-01 does not carry the *AVR-Rmg8* gene, wheat cultivars and breeding materials with *Rmg8* as a source of resistance would likely be

susceptible. Two of the isolates collected in Brazil in 2017 from infected wheat materials carrying the 2NS translocation, were also negative for *AVR-Rmg8*. This implies that these new isolates would break two of the most important sources of resistance identified. Wang et al. (2018) reported the new resistance gene *RmgGR119*, identified in the accession GR119, which also carries the *Rmg8* gene. According to the authors, the resistance observed in GR119 results from the additive effects of both genes, since typical lesions were observed in detached leaves inoculated with an isolate with the *AVR-Rmg8* disrupted (Br48ΔA8_d6). Taking these considerations together, it is of great importance to maintain a constant evaluation of the distribution of *AVR* genes in MoT populations, as an indicator of the changes they may be experiencing. It is also key to identify and clone AVR genes in MoT, such as AVR- *RmgGR119.* The incorporation of resistance genes into elite materials requires a lot of time and effort, therefore, pathogen populations should be monitored to identify the most appropriate sources of resistance to be used.

Homologs to MoO effector genes *AVR-Piz-t, AVR-Pi9*, *AVR-Pi54, ACE1*, *BAS2*, *BAS3* and *BAS4* were the most frequently amplified in MoT isolates. Although the function of BAS2, BAS3 and BAS4 is not clearly understood, these small secreted proteins are thought to be related to the biotrophic invasion of the host during compatible (susceptible) interactions (Mosquera et al. 2009). The authors' results suggested that BAS2 can be a structural component of the biotrophic interfacial complex (BIC) or an effector that is translocated into the rice cells, while BAS3 may be involved in cell-to-cell movement of the invasive hyphae (IH), and BAS4 may be an interfacial matrix protein (Mosquera et al. 2009). *AVR-Piz-t, AVR-Pi9*, *AVR-Pi54,* and *ACE1* encode avirulence (AVR) effectors that are recognized by cognate rice-resistance genes (R), triggering resistance in incompatible interactions. *AVR-Piz-t* encodes a small secreted protein of

108 amino-acids (aa) with no homology to any other reported protein (Li et al. 2009). Insertion of transposons within the promoter region, or a single nucleotide substitution, resulting in an amino-acid change, can cause the loss-of-function of *AVR-Piz-t* (Li et al. 2009, Li et al. 2012). *Piz-t* is the cognate R gene of *AVR-Piz-t* in rice, and it encodes a 1033 aa protein, belong to the NBS-LRR (Nucleotide Binding Site Leucine-Rich Repeats or NLR) class of resistance proteins (Zhou et al. 2006). *Piz-t* and *AVR-Piz-t* interacts indirectly (Park et al., 2012; Park et al., 2016; Wang et al., 2016), and this will be discussed later. *AVR-Pi9* encodes a small secreted protein of 91 aa that localizes in the BIC and is translocated into the host cell, the gene is located in a genomic region proximal to the putative centromere of chromosome 7 (Wu et al., 2015). The cognate resistance gene *Pi9* encodes a NBS-LRR protein of 1032 aa, and the gene is constitutively expressed in rice plants (Qu et al. 2006). *Piz-t* and *Pi9* were mapped at the same locus near the centromere of the chromosome 6, and they are sequence-related, with only eight amino acids that differentiate them (Zhou et al., 2006), but there is not sequence similarity between the cognate *AVR* genes, *AVR-Piz-t* and *AVR-Pi9* (Wu et al., 2015). The type of interaction between Pi9 and AVR-Pi9 has not been clarified. *AVR-Pi54* encodes a secreted protein of 153 aa (Ray et al., 2016); its cognate resistance gene *Pi54* encodes a NLR protein of 330 aa, whose expression is induced by pathogen infection (Sharma et al., 2005; Sharma et al., 2010). Pi54 interacts directly with AVR-Pi54 and induces hypersensitive cell death in rice (Ray et al, 2016). *ACE1* encodes a large cytoplasmic enzyme of 4035 aa (Avirulence Conferring Enzyme 1) that is a hybrid between a polyketide synthase (PKS) and a nonribosomal peptide synthetase (NRPS) (Böhnert et al., 2004). ACE1 is expressed during penetration and localized in the cytoplasm of the appressorium, and likely it is involved in the biosynthesis of a secondary metabolite that function as an effector (Böhnert et al., 2004). The cognate resistance gene *Pi33*

has not been cloned, but it has been mapped on rice chromosome 8 in a 240 kb region (Berruyer, et al., 2003; Raboin et al., 2016). Due to the high frequency of occurrence of these *AVR* genes in MoT isolates, the incorporation of the cognate resistance genes in wheat by biotechnological tools may be a valuable alternative and more efficient way to introduce resistance to wheat blast.

In this study, wheat embryogenic calli were co-transformed with the rice resistance gene *Piz-t* and the *bar* gene, which confers resistance to the herbicide. Using molecular analysis, 22 plants carrying the *bar* gene were identified, but only 11 of these also had the *Piz-t* gene. This represents a frequency of co-transformation of 50%, which is low considering the previous experiments performed with AMPs (76%, Chapter 2) and other reports where the frequencies were more than 85% (Stoger et al., 1998; Tian et al., 2019). Co-transformation usually results from the co-integration of both plasmids as arrays in the same locus, but this occurs after the formation of plasmid-plasmid junctions (Kohli et al., 1998; Kohli et al., 1999). It is possible that the low co-transformation observed was due to the low homology between the plasmids used. pPiz-t_C1305 was constructed using the pCAMBIA1305, which is designed for *Agrobacterium*-mediated plant transformation; this could limit the formation of the plasmid-plasmid junctions before integration. The high number of plants only carrying the *bar* gene suggests that the integration of plasmids was successful. Expression analysis showed co-expression of *Piz-t* and *bar* genes in T_0 transgenic plants but silencing of *Piz-t* was observed in some T_1 plants whose seeds came from specific tillers. For example, silencing was observed in T_1 plants of the line Piz-t_4739.A (seeds from tiller A), but not in plants from Piz-t_4739.C (tiller C); likewise, *Piz-t was* silenced in T_1 plants from Piz-t_8212.B (tiller B) but not in T_1 plants from Piz-t_8212.A (tiller A). Since the tillers were developed from the same T_0 plant, it is likely that silencing occurred during the formation of the seeds. It is also possible that the silencing was due to promoter

methylation, rather than due to the effects of the position of the transgene or copy number. Co-segregation of *Piz-t* and *bar* was observed in the T_1 and following generations in almost all transgenic lines, with exception of Piz-t_8210, in which the transgene *Piz-t* was not detected in plants from the T_1 generation, but *bar* was inherited until generation T_3. The copy number of the *Piz-t* in the T_0 plants, measured by using ddPCR, ranged from one copy to 19 copies (Figure 3.2). Five plants had a single copy (46%), 4 plants had 2-3 copies (36%), and only 2 plants had more than 5 copies (18%). These results were consistent with those reported in other studies that also used particle bombardment to generate transgenic wheat, where the highest percentage of plants had a single copy, while only a low percentage had more than 5 copies (Blechl et al., 1998; Rasco-Gaunt et al., 2001). The relative expression of *Piz-t* in T_0 transgenic plants showed high variation (Figure 3.2), and factors like copy number of the transgene, location of the integration site, and effects of DNA methylation can been associated with this variation (Butaye et al., 2005). Some authors reported that levels of expression of transgenes are associated with the copy number, whereas other reported no correlation (Hobbs, et al., 1993; Maqbool & Christou, 1999; Stoger et al., 1998), likewise, it has been reported that the type of promoter used is the most critical factor affecting transgene expression (Jackson et al., 2001; Chen et al. 1999) . In this study, correlation between the number of copies of the transgene and the level of expression was not observed, and *Piz-t* was under the control of the rice endogenous promoter in all transgenic plants, then it is likely that expression levels are associated with transgene integration sites.

The response of seven transgenic lines expressing *Piz-t* to head infection was evaluated. T_2 and T_3 plants from the transgenic lines Piz-t_5238.C.1 and Piz-t_5503.C1 showed, in three independent experiments, a slight reduction in the percentage of affected spikelets when

compared to other transgenic lines and controls (Figure 3.3 and Figure 3.5), nevertheless, the percentages of affected spikelets were always higher than 82% on the last day of evaluation (Figure 3.5). In leaf infection assays, seedlings from these two lines, and from the line Piz-t_8212.B, showed a reduction in the percentage of affected area (Figure 3.6). Seedlings from Piz-t_5238.C.1 and Piz-t_5503.C.1 were re-assessed, and they showed a significant reduction in the percentage of affected area when compared to controls. As previously reported by Cruz et al. (2012), there was not a strong correlation between the response of seedlings and heads to MoT. The results suggest that the expression of *Piz-t* in leaves may confer some resistance to MoT infection, however, the results were very variable. For example, in the first bioassay, plants from lines Piz-t_5238.C and Piz-t_5503.C showed 44% and 37% less affected area than the control Bobwhite_WT, respectively. However, in the following bioassay, the reductions were 22% for Piz-t_5238.C (30.88% versus 39.63% in Bobwhite_WT), and 54% for Piz-t_5503.C (18.13% versus 39.63 in Bobwhite_WT). These variations may be due to the inoculation process, since the distribution of inoculum using a sprayer is not homogeneous in all the leaves. An improved methodology that ensures a homogeneous distribution of inoculum in all the leaves should be developed to validate these results and increase reproducibility when evaluating W_LB.

Incompatible reactions involving resistance and avirulence genes usually result in a hypersensitive response of the plant to limit the development of the pathogen, although this kind of response was not observed in plants expressing *Piz-t*, neither in heads nor on the leaves. This may be due to the fact that, as mentioned above, the interaction between Piz-t and AVR-Piz-t is not direct, and other proteins must be required to mediate the resistance response. In rice, AVR-Piz-t interacts with 12 APIP (AVR-Piz-t Interacting Proteins) (Park et al., 2012), two of these proteins, APIP6 and APIP10 are functional ring E3 ubiquitin ligases involved in the regulation of

basal defense (pathogen-associated molecular pattern-triggered immunity or PTI). In addition, APIP10 acts as a negative regulator of Piz-t to avoid cell death due to accumulation of the protein (Park et al., 2012; Park et al., 2016). When *M. oryzae* infects rice plants without the R gene *Piz-t*, AVR-Piz-t functions as a virulence effector suppressing the basal defense (PTI), by interacting and promoting the degradation of APIP6/APIP10 (Park et al., 2012; Park et al., 2016). However, when *M. oryzae* infects rice plants that contain *Piz-t*, degradation of APIP10 removes the negative control over *Piz-t*, and the protein accumulation results in cell death and resistance (Park et al., 2012; Park et al., 2016). In addition, during the necrotrophic stage, AVR-Piz-t interacts directly with APIP5 (a bZIP-type transcription factor) suppressing its transcriptional activity and promoting the cell death (effector-triggered necrosis); but in plants carrying *Piz-t*, the protein stabilizes APIP5 and prevents cell necrosis (Wang et al. 2016). Then, the resistance response mediated by Piz-t requires the action of at least three additional host proteins that directly interact with the AVR protein. The rice blast pathogen secretes AVR-Piz-t to suppress the basal defense during the biotrophic stage (Park et al. 2012), but although it is known that the MoT isolate T-25 has the functional allele (M. Farman, personal communication), the expression of this gene during wheat infection should be confirmed. Likewise, the presence of orthologs of APIP6/APIP10/APIP5 in wheat must be evaluated. Two wheat RING-type domain-containing proteins (Uniprot accessions A0A3B5YUY7, A0A3B5Z36) and an uncharacterized protein (accession A0A3B5XW01) share about 85% identity with the rice protein APIP6 (Uniprot accession Os05g0154600), and two coding regions in the 5AS and 5DS wheat chromosomes (BZIP domain-containing predicted proteins, accessions A0A3B6KBK6 and A0A3B6MJX8) share 77% of homology with the rice *APIP5* gene (accession number LOC_Os06g50310). Detailed studies should be conducted to determine their potential interaction

with ARV-Piz-t. In rice, levels of the protein Piz-t are regulated by APIP10 (ring finger E3 ligase), and the accumulation of the protein due to the silencing of APIP10 results in spontaneous necrosis, indicating the role of Piz-t in programmed cell death (Park et al. 2016). The expression of *Piz-t* in leaves and heads of transgenic wheat (confirmed up to the messenger RNA level) did not induce cell death, suggesting that (i) the protein was not able to activate this type of response in wheat, and/or (ii) there is a functional ortholog(s) of APIP10 in wheat that regulates the level of the protein. There are still many questions about why the type of resistance expected was not observed in Piz-t transgenic lines. Other rice resistance genes could be better candidates than *Piz-t* to try to incorporate blast resistance in wheat, such as *Pi54*, which has a direct interaction with the cognate *AVR-Pi54* (Ray et al., 2016), or the *Pi-9* gene, although the type of interaction with *AVR-Pi9* is not known. However, it must be assessed previously whether the alleles *AVR-Pi9* and *AVR-Pi54* in MoT populations are functional, inducing the response of the cognate resistance genes.

Wheat transgenic plants expressing AMPs were also challenged with MoT isolate T-25. Of the 7 lines exposed to head infection, plants form 3 lines Wj1_8538.C.2 (T_2 and T_3 plants) Wj1_8650.C.3 and Ace1_8666.A.1 showed slight reductions in the percentage of affected spikelets during the first days of evaluation (Figure 3.3), and significant differences in the AUDPC were detected for Wj1_8538.C.2 and Wj1_8650.C.3. Nevertheless the percentage of affected area for these plants at the end of the evaluation was higher than 82%, and they were considered susceptible. Significant differences in the AUDP were found in lines ARC1_8894.D.1.4 and Zma_8558.A.5.4, but for both lines, the percentage of affected area 14 dai was 98%. Two of the 7 lines evaluated in leaf assays, Wj1_8582.C.3 and Wj1_8650.C.3, showed reduction in the percentage of leaf affected area in the disease progress curve (Figure 3.4

B), but although the difference in the AUDPC of Wj1_8650.C.3 was significant, the percentage of leaf affected in both lines was around 41% versus 47.5% observed in Bobwhite_WT. These results indicate that the AMPs used in this study did not confer resistance to W_SB or W_LB. These results contrast with some reports where the AMPs Ace-AMP1 and WD (Wj1) were used to transform rice plants, and resistance to rice blast was observed. Patkar & Chattoo (2006) reported that the expression of *Ace-AMP1* in rice enhanced the resistance to blast by 86%, diseased leaf area (DLA) in transgenic lines ranged from 0.97 to 14.11%, whereas untransformed plants had 47.65% DLA. In addition, authors reported that hyphal growth was inhibited or the hyphae showed distorted morphology after 2 to 3 days of inoculation in transgenic plants. Kanzaki et. al (2002) found that rice plants expressing the wasabi defensin gene (*WD*) showed different levels of resistance to the rice blast fungus; some plants (T_2) were as susceptible as the susceptible control, while others were as resistant as the resistant control. The two lines with greater resistance showed reduction in the number and size of the lesions, and the progeny of these two lines (T_3 plants) showed a 50% reduction in the size of the lesions. In this study, AMP genes were under control of the constitutive promoter *Ubi1*, and gene expression was confirmed in leaves and heads up to the level of messenger RNA (mRNA), however due to the unavailability of antibodies against these AMPs, the synthesis of proteins in transgenic plants was not evaluated. An alternative to determine if transgenic plants are producing biologically active AMPs is to evaluate the effect of crude protein extracts obtained from these plants on the *in vitro* growth of MoT.

This is the first study in which the presence of effector genes is evaluated in a significant group of *M. oryzae Triticum* pathotype isolates. The results suggested that the distribution of effectors is changing in the populations, and these changes must be considered by breeding

programs, as they can have a strong impact on the durability of resistance. This is also the first report in which a rice blast resistance gene, and genes encoding AMPs were used to try to reduce susceptibility to wheat blast. None of the transgenic lines showed resistance to WsB, while two lines expressing the rice resistance gene *Piz-t* showed a reduction in W_LB severity. Other rice resistance genes such as *Pi54* and *Pi9* would be good candidates to try to incorporate resistance into wheat, as the cognate *AVR* genes are widely distributed in MoT populations.

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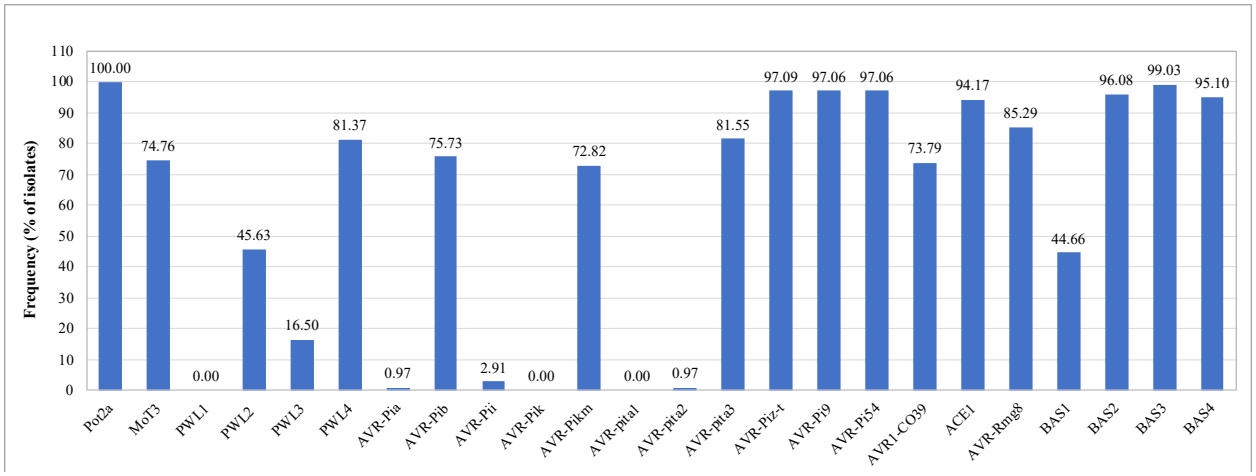
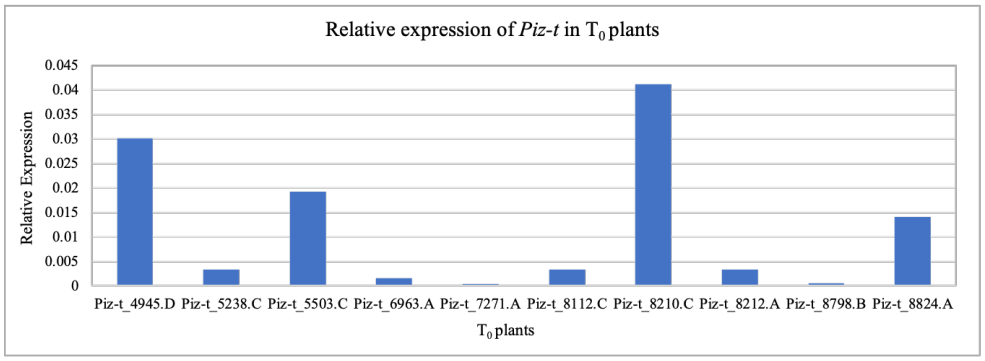


Figure 3.1 Frequency of occurrence of twenty-one homologs of MoO effector genes and the *AVR-Rmg8* in 102 MoT isolates.

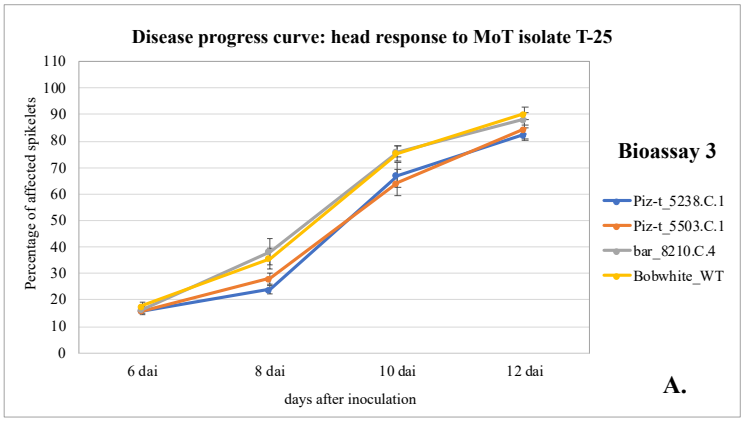
Markers Pot2 for *M. oryzae* from different hosts, and MoT3 for *M. oryzae Triticum* pathotype were also included.

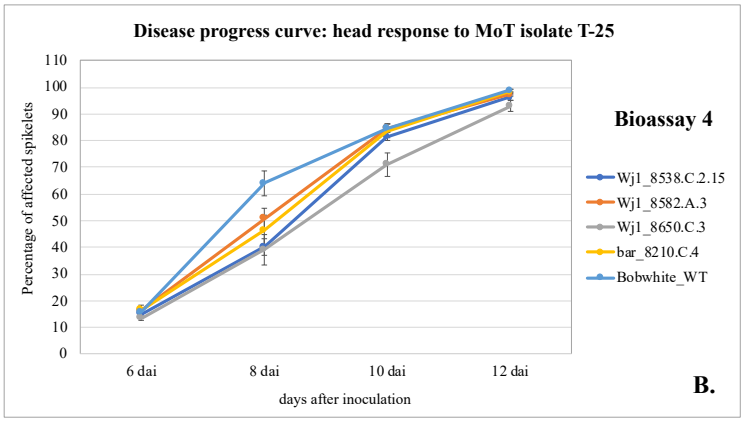


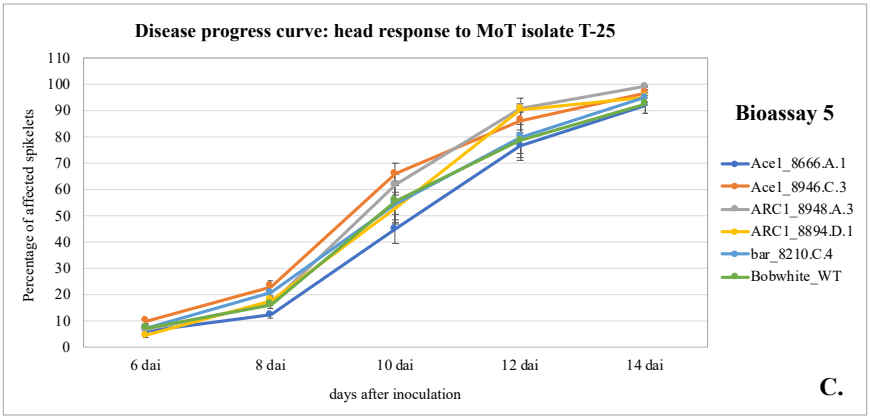
T_0 plant	Estimated number of copies
Piz-t_4739.C	0.95
Piz-t_4945.D	1.16
Piz-t_5238.C	18.86
Piz-t_5503.C	3.42
Piz-t_6963.A	3.14
Piz-t_7271.A	0.92
Piz-t_8112.C	6.06
Piz-t_8210.C	0.99
Piz-t_8212.A	2.63
Piz-t_8798.B	0.97
Piz-t_8824.A	1.99

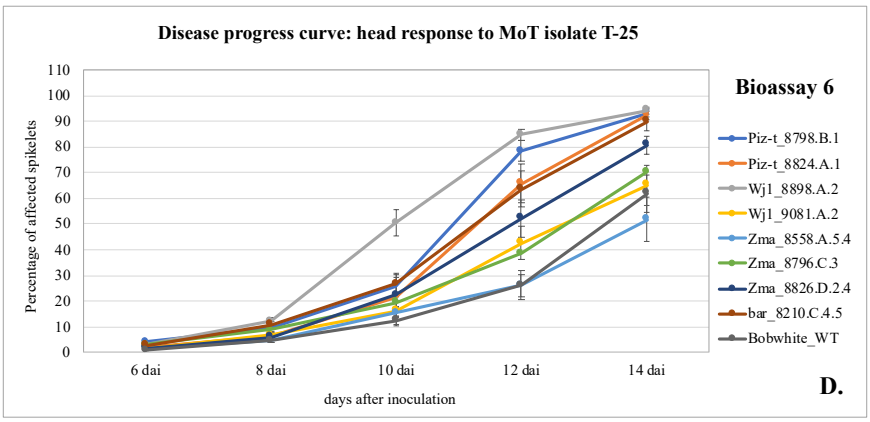
Figure 3.2 Relative expression and number of copies of *Piz-t* in T_0 plants.

Relative expression was estimated by RT-qPCR using the ΔCt method, with *actin* as reference gene; the copy number was estimated by ddPCR using *Puroindoline-b* (*Pinb*) as reference gene.


Disease progress curve: head response to MoT isolate T-25
Percentage of affected spikelets
110
100
90
80
70
60
50
40
30
20
10
0
6 dai
8 dai
10 dai
12 dai
days after inoculation
Bioassay 3
Piz-t_5238.C.1
Piz-t_5503.C.1
bar_8210.C.4
Bobwhite_WT
A.


Disease progress curve: head response to MoT isolate T-25
Percentage of affected spikelets
110
100
90
80
70
60
50
40
30
20
10
0
6 dai
8 dai
10 dai
12 dai
days after inoculation
Bioassay 4
Wj1_8538.C.2.15
Wj1_8582.A.3
Wj1_8650.C.3
bar_8210.C.4
Bobwhite_WT
B.


Disease progress curve: head response to MoT isolate T-25
Percentage of affected spikelets
110
100
90
80
70
60
50
40
30
20
10
0
6 dai
8 dai
10 dai
12 dai
14 dai
days after inoculation
Bioassay 5
Ace1_8666.A.1
Ace1_8946.C.3
ARC1_8948.A.3
ARC1_8894.D.1
bar_8210.C.4
Bobwhite_WT
C.


Disease progress curve: head response to MoT isolate T-25
Percentage of affected spikelets
110
100
90
80
70
60
50
40
30
20
10
0
6 dai
8 dai
10 dai
12 dai
14 dai
days after inoculation
Bioassay 6
Piz-t_8798.B.1
Piz-t_8824.A.1
Wj1_8898.A.2
Wj1_9081.A.2
Zma_8558.A.5.4
Zma_8796.C.3
Zma_8826.D.2.4
bar_8210.C.4.5
Bobwhite_WT
D.

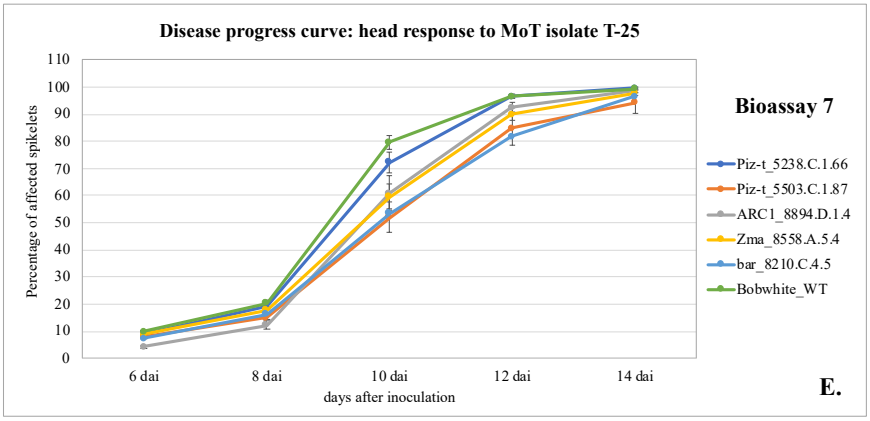


Figure 3.3 Disease progress curves of the bioassays evaluating the head response to MoT. Figure A: bioassay 3, B: bioassay 4, C: bioassay 5, D: bioassay 6, and E: bioassay 7. The response of plants from the transgenic lines and controls to MoT was evaluated as the percentage of spikelets affected scored at different time points (days after inoculation). Error bars represent the standard error.

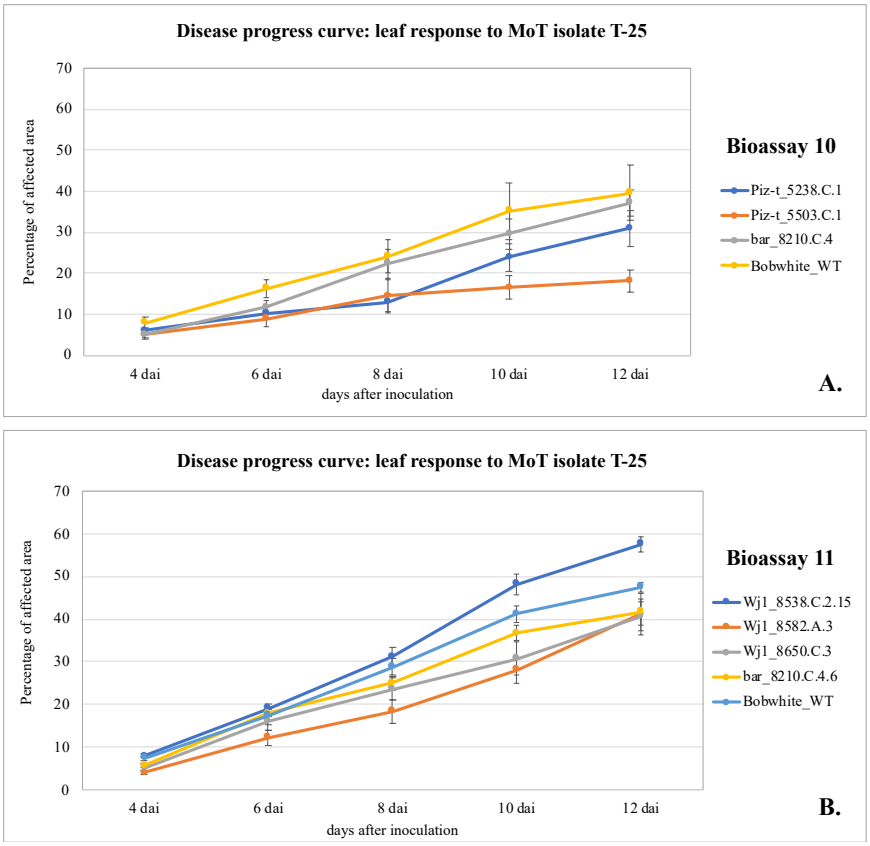
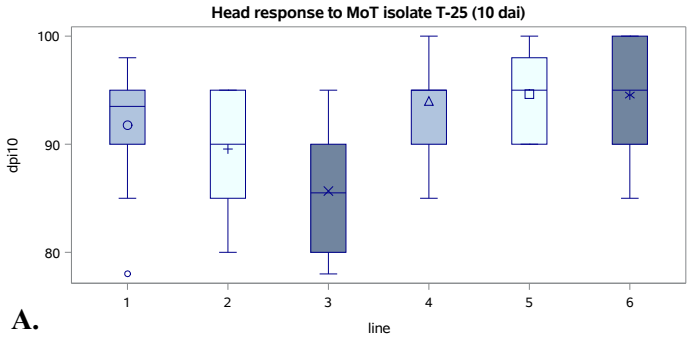


Figure 3.4 Disease progress curves of the bioassays evaluating the leaf response to MoT. Figure A: bioassay 10, B: bioassay 11. The response of plants from the transgenic lines and controls to MoT was evaluated as the percentage of leaf area affected scored at different time points (days after inoculation). Error bars represent the standard error.

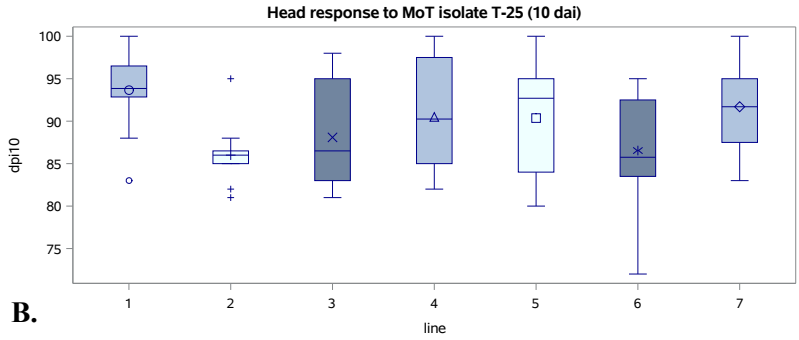


A.

Bioassay 1

	Line	10 dai	
1	Ace1_8476.B.6	91.75	ab
2	ARC1_8524.C.9	89.56	ab
3	Wj1_8538.C.2	82.00	a
4	Zma_8558.A.5	94.00	b
5	bar_8210.C.4	93.17	b
6	Bobwhite_WT	93.33	b

P > F 0.0081

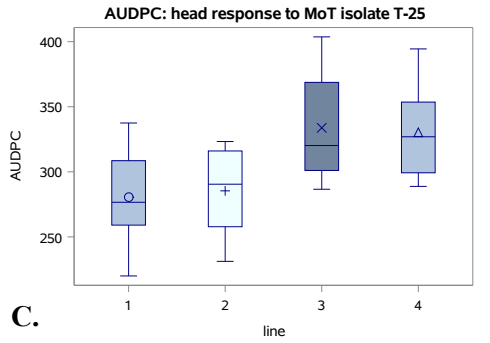


B.

Bioassay 2

	Line	10 dai	
1	Piz-t_4945.D.1	93.68	b
2	Piz-t_5238.C.1	86.00	a
3	Piz-t_5503.C.1	88.09	ab
4	Piz-t_8112.C.1	90.50	ab
5	Piz-t_8212.A.1	90.37	ab
6	bar_8210.C.4	86.54	a
7	Bobwhite_WT	91.70	ab

P > F 0.0192

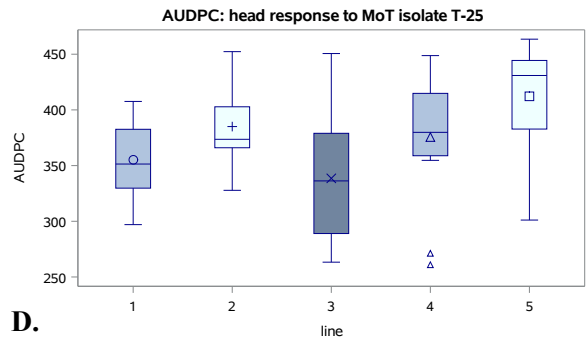


C.

Bioassay 3

	Line	12 dai♠	AUDPC♣	
1	Piz-t_5238.C.1	82.59	280.73	a
2	Piz-t_5503.C.1	84.60	285.39	ab
3	bar_8210.C.4	88.35	333.78	c
4	Bobwhite_WT	90.44	330.34	bc

P > F 0.0114

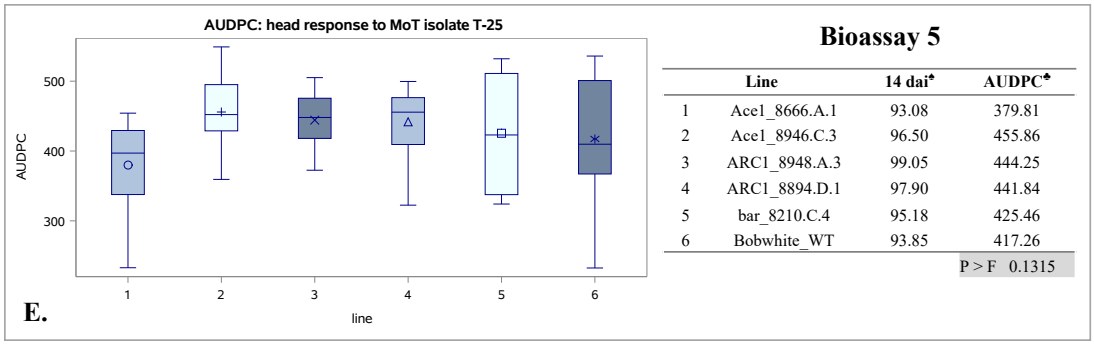


D.

Bioassay 4

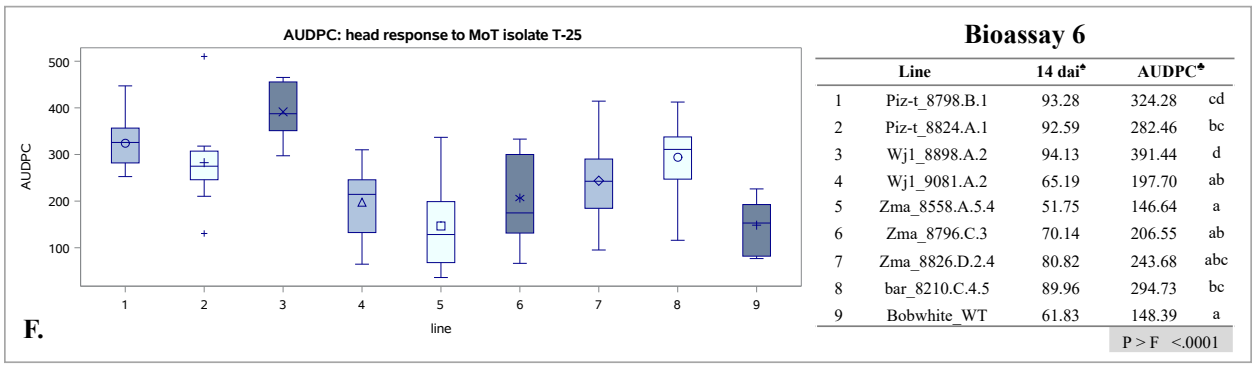
	Line	12 dai♠	AUDPC♣	
1	Wj1_8538.C.2.15	96.33	355.03	a
2	Wj1_8582.A.3	97.35	385.04	ab
3	Wj1_8650.C.3	94.47	338.55	a
4	bar_8210.C.4	98.24	375.64	ab
5	Bobwhite_WT	98.83	412.19	b

P > F 0.0062



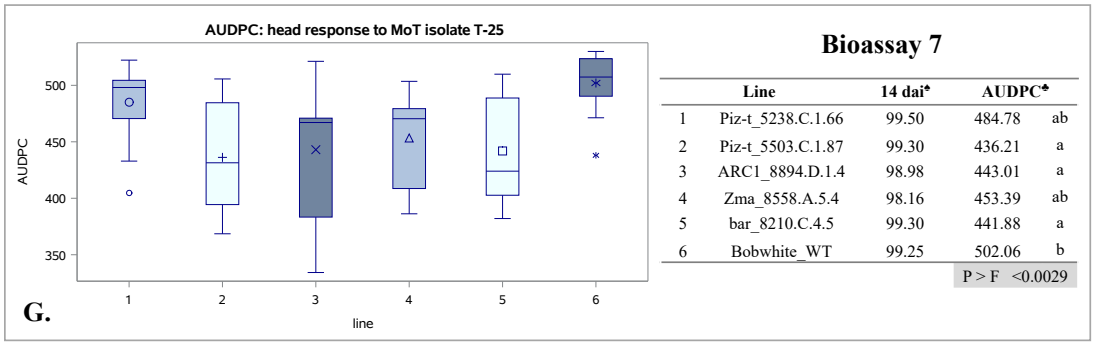
Bioassay 5

	Line	14 dai♠	AUDPC♣
1	Ace1_8666.A.1	93.08	379.81
2	Ace1_8946.C.3	96.50	455.86
3	ARC1_8948.A.3	99.05	444.25
4	ARC1_8894.D.1	97.90	441.84
5	bar_8210.C.4	95.18	425.46
6	Bobwhite_WT	93.85	417.26
			P > F 0.1315



Bioassay 6

	Line	14 dai♠	AUDPC♣	
1	Piz-t_8798.B.1	93.28	324.28	cd
2	Piz-t_8824.A.1	92.59	282.46	bc
3	Wj1_8898.A.2	94.13	391.44	d
4	Wj1_9081.A.2	65.19	197.70	ab
5	Zma_8558.A.5.4	51.75	146.64	a
6	Zma_8796.C.3	70.14	206.55	ab
7	Zma_8826.D.2.4	80.82	243.68	abc
8	bar_8210.C.4.5	89.96	294.73	bc
9	Bobwhite_WT	61.83	148.39	a
			P > F <.0001	



Bioassay 7

	Line	14 dai♠	AUDPC♣	
1	Piz-t_5238.C.1.66	99.50	484.78	ab
2	Piz-t_5503.C.1.87	99.30	436.21	a
3	ARC1_8894.D.1.4	98.98	443.01	a
4	Zma_8558.A.5.4	98.16	453.39	ab
5	bar_8210.C.4.5	99.30	441.88	a
6	Bobwhite_WT	99.25	502.06	b
			P > F <0.0029	

Figure 3.5 Response of heads to MoT at specific evaluation time points (10 dai for A: bioassay 1, and B: bioassay 2), or using the AUDPC (C: bioassay 3, D: bioassay 4, E: bioassay 5, F: bioassay 6, and G: bioassay 7).

AUDPC was calculated using the trapezoidal method proposed by Madden et al. (2007).

♠ mean percentage of spikelets affected scored on the last evaluation day (12 or 14 dai).

♣AUDCP means were analyzed using the GLM procedure (SAS v.3.8), with a significance level of 5% (α= 0.05). When significant differences were observed, the means were compared with the Ryan-Einot-Gabriel-Welsch F (REGWF) multiple comparison test. AUDPC means with the

same letter are not significantly different. Response to MoT at a specific time point () or using the AUDPC.

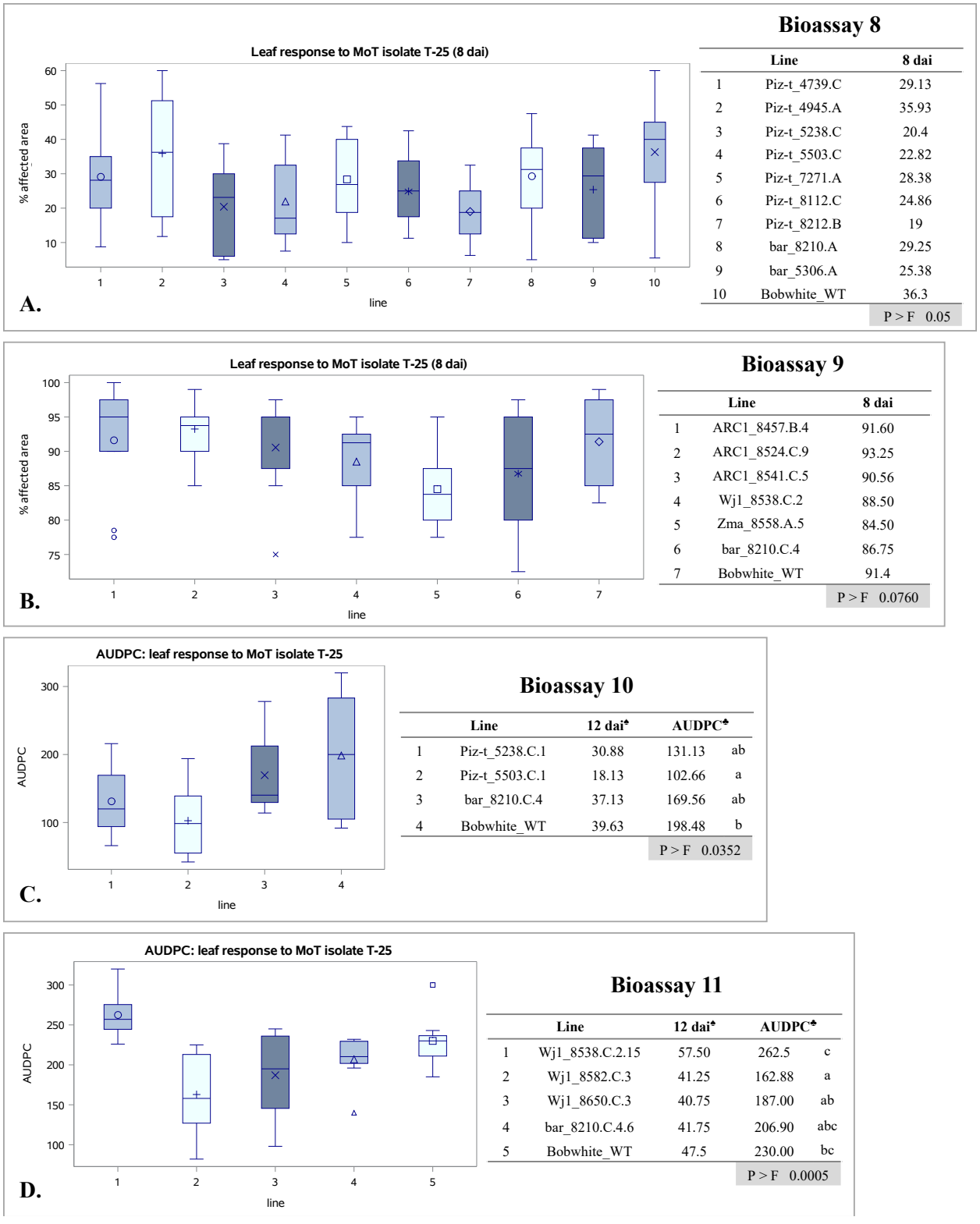


Figure 3.6 Response of leaves to MoT at a specific time point (8 dai for A: bioassay 8, and B: bioassay 9), or using the AUDPC (C: bioassay 10, and D: bioassay 11).

AUDPC was calculated using the trapezoidal method proposed by Madden et al. (2007).

♠ mean percentage of spikelets affected scored on the last evaluation day (12 or 14 dai).

♣AUDCP means were analyzed using the GLM procedure (SAS v.3.8), with a significance level of 5% (α= 0.05). When significant differences were observed, the means were compared with the Ryan-Einot-Gabriel-Welsch F (REGWF) multiple comparison test. AUDPC means with the same letter are not significantly different.

Table 3.1 *M. oryzae* (*Triticum*, *Lolium* and *Digitaria* pathotypes) isolates used in this study.

Isolate	FDWSRU no.	Collection Year	Country	Location	Host
T-3	BR86Ta003	1986	Brazil	Vicentinopolis-GO	*Triticum aestivum*
T-50	BR86Ta050	1986	Brazil		
T-2	BR87Ta002	1987	Brazil	Londrina PR	*Triticum aestivum*
T-4	BR88Ta004	1988	Brazil	Floresta,Parana	*Triticum aestivum*
T-5	BR88Ta005	1988	Brazil	Palotina, Parana	*Triticum aestivum*
T-6	BR88Ta006	1988	Brazil	Fenix, Parana	*Triticum aestivum*
T-7	BR88Ta007	1988	Brazil	Cianorte, Parana	*Triticum aestivum*
T-9	BR88Ta009	1988	Brazil	Maringa,PS Moacir Ferro Farm	*Triticum aestivum*
T-12	BR88Ta012	1988	Brazil	Floresta,PS Pallaro Farm	*Triticum aestivum*
T-15	BR88Ta015	1988	Brazil	Floresta,PS Lindolfo Farm	*Triticum aestivum*
T-17	BR88Ta017	1988	Brazil	Maringa,PS Moacir Ferro Farm	*Triticum aestivum*
T-21	BR88Ta021	1988	Brazil	Floresta,PS Lindolfo Farm	*Triticum aestivum*
T-22	BR88Ta022	1988	Brazil	Floresta,PS Pallaro Farm	*Triticum aestivum*
T-25	BR88Ta025	1988	Brazil	Sao Jorge Do Ivar	*Triticum aestivum*
T-34	BR89Ta034	1989	Brazil	Panema	*Triticum aestivum*
T-37	BR89Ta037	1989	Brazil	Cianorte	*Triticum aestivum*
T-42	BR89Ta042	1989	Brazil	Cianorte	*Triticum aestivum*
Py 5003	BR05Ta001	2005	Brazil	Londrina - PR	*Triticum aestivum*
Py 6047	BR06Ta412	2006	Brazil	Goiânia - GO	*Triticum aestivum*
Py 25.1	BR07Ta030	2007	Brazil	Palotina - PR	*Triticum aestivum*
Py 35.3	BR07Ta051	2007	Brazil	Brasília -DF (PAD)	*Triticum aestivum*
Py 86.1	BR08Ta416	2008	Brazil	Cascavel - PR	*Triticum aestivum*
Py 12.1.013	BR12Ta395	2012	Brazil	Patrocínio - MG	*Triticum aestivum*
Py 12.1.014	BR12Ta396	2012	Brazil	Patrocínio - MG	*Triticum aestivum*
Py 12.1.062	BR12Ta397	2012	Brazil	Rio Verde (2) - GO	*Triticum aestivum*
Py 12.1.063	BR12Ta398	2012	Brazil	Rio Verde (2) - GO	*Triticum aestivum*
Py 12.1.065	BR12Ta399	2012	Brazil	Rio Verde (2) - GO	*Triticum aestivum*
Py 12.1.147	BR12Ta401	2012	Brazil	Amambai (1) - MS	*Triticum aestivum*
Py 12.1.347	BR12Ta402	2012	Brazil	Aral Moreira (3) - MS	*Triticum aestivum*
Py 12.1.206	BR12Ta408	2012	Brazil	São Borja – RS	*Triticum aestivum*
Py 12.1.207	BR12Ta409	2012	Brazil	São Borja – RS	*Triticum aestivum*
16-Mot-01	BR16TA145	2016	Brazil	Passo Fundo/RS	*Triticum aestivum*
16-Mot-02	BR16TA146	2016	Brazil	Passo Fundo/RS	*Triticum aestivum*
16-Mot-04	BR16TA147	2016	Brazil	Passo Fundo/RS	*Triticum aestivum*
16-MoL-05	BR16LA148	2016	Brazil	Porto Uniao/SC	*Lolium multiflorum*
16-Mot-08	BR16TA150	2016	Brazil	Santa Rosa/RS	*Triticum aestivum*
16-Mot-09	BR16TA151	2016	Brazil	Santa Rosa/RS	*Triticum aestivum*
16-Mot-10	BR16TA152	2016	Brazil	Santa Rosa/RS	*Triticum aestivum*
16-Mot-11	BR16TA153	2016	Brazil	Santa Rosa/RS	*Triticum aestivum*
16-Mot-12	BR16TA154	2016	Brazil	Santa Rosa/RS	*Triticum aestivum*
16-MoL-14	BR16LA156	2016	Brazil	Santa Rosa/RS	*Lolium multiflorum*
16-Mot-15	BR16TA157	2016	Brazil	Santa Rosa/RS	*Triticum aestivum*
16-Mot-17	BR16TA158	2016	Brazil	Santa Rosa/RS	*Triticum aestivum*
16-Mot-19	BR16TA159	2016	Brazil	Santa Rosa/RS	*Triticum aestivum*
16-Mot-22	BR16TA160	2016	Brazil	Santa Rosa/RS	*Triticum aestivum*
16-Mot-23	BR16TA161	2016	Brazil	Santa Rosa/RS	*Triticum aestivum*
16-Mot-27	BR16TA162	2016	Brazil	Santa Rosa/RS	*Triticum aestivum*
16-Mot-28	BR16TA163	2016	Brazil	Santa Rosa/RS	*Triticum aestivum*
16-Mot-30	BR16TA164	2016	Brazil	Santa Rosa/RS	*Triticum aestivum*

Isolate	FDWSRU no.	Collection Year	Country	Location	Host
16-Mot-31	BR16TA165	2016	Brazil	Santa Rosa/RS	*Triticum aestivum*
16-Mot-32	BR16TA166	2016	Brazil	Santa Rosa/RS	*Triticum aestivum*
16-Mot-33	BR16TA167	2016	Brazil	Santa Rosa/RS	*Triticum aestivum*
16-MoT-34		2016	Brazil	Coxilha/RS	*Triticum aestivum*
16-Mot-35	BR16TA168	2016	Brazil	Coxilha/RS	*Triticum aestivum*
16-Mot-36	BR16TA169	2016	Brazil	Coxilha/RS	*Triticum aestivum*
17-Mot-37	BR17TA170	2017	Brazil	Nao-me-Toque/RS	*Triticum aestivum*
17-Mot-38	BR17TA171	2017	Brazil	Cascavel/PR	*Triticum aestivum*
17-Mot-39	BR17TA172	2017	Brazil	Cascavel/PR	*Triticum aestivum*
17-MoD-40	BR17DG173	2017	Brazil	Cascavel/PR	*Digitaria horizontalis*
17-Mot-41	BR17TA174	2017	Brazil	Cascavel/PR	*Triticum aestivum*
17-Mot-42	BR17TA175	2017	Brazil	Cascavel/PR	*Triticum aestivum*
17-Mot-43	BR17TA176	2017	Brazil	Nao-me-Toque/RS	*Triticum aestivum*
17-Mot-45	BR17TA177	2017	Brazil	Nao-me-Toque/RS	*Triticum aestivum*
17-Mot-46	BR17TA178	2017	Brazil	Nao-me-Toque/RS	*Triticum aestivum*
17-MoT-47	BR17TA179	2017	Brazil	Nao-me-Toque/RS	*Triticum aestivum*
B-1		2011	Bolivia	Quirusillas	*Triticum aestivum*
B-2	BO11Ta001	2011	Bolivia	Quirusillas	*Triticum aestivum*
B-4	BO11Ta003	2011	Bolivia	Quirusillas	*Triticum aestivum*
B-8	BO11Ta007	2011	Bolivia	Quirusillas	*Triticum aestivum*
B-17	BO11Ta009	2011	Bolivia	Quirusillas	*Triticum aestivum*
B-30	BO11Ta010	2011	Bolivia	Quirusillas	*Triticum aestivum*
B-52	BO12Ta019	2012	Bolivia	Quirusillas	*Triticum aestivum*
B-53	BO12Ta020	2012	Bolivia	Quirusillas	*Triticum aestivum*
B-59	BO12Ta026	2012	Bolivia	Quirusillas	*Triticum aestivum*
B-60	BO12Ta027	2012	Bolivia	Quirusillas	*Triticum aestivum*
B-61	BO12Ta028	2012	Bolivia	Quirusillas	*Triticum aestivum*
BRI-B70	BO12Ta300	2012	Bolivia	Santa Cruz, Pailon (La Castaña Farm)	*Triticum aestivum*
BRI-B71	BO12Ta029	2012	Bolivia	Santa Cruz, Okinawa 2 (Centro Exp. CAICO)	*Triticum aestivum*
BRI-B72	BO12Ta301	2012	Bolivia	Santa Cruz, Pailon (La Castaña Farm)	*Triticum aestivum*
BRI-B73	BO12Ta302	2012	Bolivia	Santa Cruz, Pailon (La Castaña Farm)	*Triticum aestivum*
BRI-B104	BO14Ta311	2014	Bolivia	Santa Cruz (Okinawa 2)	*Triticum aestivum*
BRI-B105	BO14Ta312	2014	Bolivia	Santa Cruz (Okinawa 2)	*Triticum aestivum*
BRI-B107	BO14Ta314	2014	Bolivia	Santa Cruz (Okinawa 2)	*Triticum aestivum*
BRI-B112	BO14Ta319	2014	Bolivia	Santa Cruz	*Triticum aestivum*
BRI-B113	BO14Ta320	2014	Bolivia	Santa Cruz	*Triticum aestivum*
BRI-B117	BO14Ta324	2014	Bolivia	Quirusillas	*Triticum aestivum*
BRI-B121	BO14Ta328	2014	Bolivia	Quirusillas	*Triticum aestivum*
BRI-B122	BO14Ta329	2014	Bolivia	Quirusillas	*Triticum aestivum*
BRI-B124	BO14Ta331	2014	Bolivia	Quirusillas	*Triticum aestivum*
BO17Ta021		2017	Bolivia		*Triticum aestivum*
P-3	PA12Td039	2012	Paraguay	Canindeyu	*Triticum durum*
P-5	PA12Ta044	2012	Paraguay	Canindeyu	*Triticum aestivum*
P-6	PA12Ta045	2012	Paraguay	Canindeyu	*Triticum aestivum*
P-8	PA12Ta047	2012	Paraguay	Canindeyu	*Triticum aestivum*
P-9	PA12Ta048	2012	Paraguay	Canindeyu	*Triticum aestivum*
P-10	PA12Ta049	2012	Paraguay	San Alberto	*Triticum aestivum*
P-11	PA12Ta050	2012	Paraguay	San Alberto	*Triticum aestivum*
P-12	PA12Ta051	2012	Paraguay	San Alberto	*Triticum aestivum*
P-14	PA12Ta053	2012	Paraguay	San Alberto	*Triticum aestivum*
P-15	PA12Ta054	2012	Paraguay	Alto Parana	*Triticum aestivum*
P-16	PA12Ta055	2012	Paraguay	Alto Parana	*Triticum aestivum*
P-17	PA12Ta056	2012	Paraguay	Alto Parana	*Triticum aestivum*
P-20	PA12Ta059	2012	Paraguay	Canindeyu	*Triticum aestivum*
P-38	PA14TA04	2014	Paraguay	Canindeyú	*Triticum aestivum*
P-40	PA18TA01	2018	Paraguay	Canindeyú	*Triticum aestivum*
KY-5506		2011	USA	Princeton, Kentucky	*Triticum aestivum*

Table 3.2 Primers used for the amplification *M. oryzae* markers and effector genes.

Primer name	Sequence (5' - 3')	PCR product size (bp)	References
Pot2a-L2	GCAATTTCATGCAACCGAAA	389	Pieck et al. (2017)
Pot2a-R2	CGTACGCCAACCAGATTGAA		
MoT3F	GTCGTCATCAACGTGACCAG	361	Pieck et al. (2017)
MoT3R	ACTTGACCCAAGCCTCGAAT		
PWL1_F1	GGTGCGGGTTCATGAGGATA	243	This study
PWL1_R1	ATATGGCAGCCCTGATCTCC		
PWL2-a	GGTGGCGGGTGGACTAAC	358	Shi et al. (2018)
PWL2-b	CCTCTTCTCGCTGTTCACGG		
PWL3_F1	TCTATTGTAAAGGGCGGGCA	245	This study
PWL3_R1	TAGTACCCATCGCCCCAATG		
PWL4_F1	GCTCCTGGTCAGCGTGATAG	210	This study
PWL4_R1	AGGACCATAGTACCCATCGC		
Avr-Pia-5	ATGCATTTTTCGACAATTTTCATC	258	Shi et al. (2018)
Avr-Pia-6	CTAGTAAGGCTCGGCAGCAAG		
Avr-Pib_F1	ATGCGTTCCTCAACCACTTT	221	This study
Avr-Pib_R1	TCCACGGTATATTTGCTGCC		
Avr-Pii-3	CCCTTTTATTCCTTCCAATTTACCA	261	Shi et al. (2018)
Avr-Pii-4	GCTTTCAGATTTTAACTTACATTAG		
Avr-Pik-a	TCACTTTGGGAACTGTCGC	267	Shi et al. (2018)
Avr-Pik-b	GGAAGTCGCCCGACAAAT		
SCO12-P1 (Pikm)	CTGTGGACTAAGTAGCATGCTTCT	946	Zhang et al. (2004)
SCO12-P2 (Pikm)	TAGGCAATCAAGAGAAAGCCAGTA		
Avr-pita1-g	GCCGAGTCGTTCTGA	475	Shi et al. (2018)
Avr-pita1-h	TGTTAATTGTGCAGAAGTTTTT		
Avr-pita2-1	TTGGCACCTTTTCATACCCAGTTT	687	Shi et al. (2018)
Avr-pita2-2	CAACTTACTTGTGAATCCCATCCC		
Avr-pita3-c	ACCGACCCAGGAAAAAAG	1797	Shi et al. (2018)
Avr-pita3-d	AAGAAACAGGCAAACGCA		
Avr-Piz-t-a	TTCACCGGGCTCGCCT	269	Shi et al. (2018)
Avr-Piz-t-b	TTCCCAATCGAGCCAACG		
Avr-Pi9_F1	GTCTTGTTCCTTGGCGTCTC	273	This study
Avr-Pi9_R1	TCTTTTCGACTTGGCACCAG		
Avr-Pi54_F2	AAATACGGGCCTGTGGTGAG	361	This study
Avr-Pi54_R2	CCCAAATCATACCCGCCACT		
Avr1-CO39-12	ATTTTGCCGCATTTTGCTAACCG	1004	Shi et al. (2018)
Avr1-CO39-13	CGACGGGCGAATCCATAGACAAG		
ACE1-23	GTTTATCTACGAGGCTGGGGACATT	1503	Shi et al. (2018)
ACE1-10	GGCGAACGGTAAAATGTAGAAGA		
Avr-Rmg8_F1	CGGGCTGTACAACATTTTCA	1450	Wang et al. (2018)
Avr-Rmg8_R1	GGAGATTTCACGATAGCAAAGC		
BAS1-F	GGTGCTTGCCACCTTTACC	300	Peng et al. (2019)
BAS1-R	TTCTCCACCCGTCTAATACCA		
BAS2_F1	TCTGTCACCGCAAATGTCAC	193	This study
BAS2_R1	CACAGCCAGTCTTGCCATTT		
BAS3_F1	CAGTTCTCCACCGTCTCCTT	336	This study
BAS3_R1	CTGAGTACGGCAAGTGGTCT		
BAS4_F1	ATGCAGCTCTCATTCTCAGC	255	This study
BAS4_R1	GCATGCGTCCTCAAACTCAT		

Table 3.3 Primers used for detection (PCR), semi-quantitative expression (RT-PCR), quantitative expression (RT-qPCR) and copy number determination (ddPCR).

Primer name	Sequence. (5'-3')	PCR product size (bp)	Description
Tub-F	ATCTGTGCCTTGACCGTATCAGG	500 gDNA / 409 cDNA	PCR and RT-PCR: control DNA contamination in cDNA samples
Tub-R	GACATCAACATTCAGAGCACCATC		
UbiABF	CCTGCCTTCATACGCTATTTATTTGC	453	PCR - *bar* detection
BarABR	CTTCAGCAGGTGGGTGTAGAGCGTG		
Bar F2	AGTCGACCGTGTACGTCTCC		RT-PCR - *bar* expression
Bar R	GAAGTCCAGCTGCCAGAAAC		
Piz-t_13F	CTGGATTGGATTGGAGCATT	712	PCR and RT-PCR - *Piz-t* detection and expression
Piz-t_13R	TGGAGAGATTGGAGGGAAGA		
qActin_2F	AGCTGGAGACTGCCAAGAAC	124	RT-qPCR - reference gene
qActin_2R	ATCATGGATGGCTGGAAGAG		
qPiz-t_1F	CTCCAAGAAAAGGCTGCTTG	95	RT-qPCR - *Piz-t* relative expression
qPiz-t_1R	TCTTGCCTAAACCACCCATC		
dd_Pinb_F1	AGTTGGCGGCTGGTACAATG	106	ddPCR - reference gene (Collier et al. 2017)
dd_Pinb_R1	ACATCGCTCCATCACGTAATCC		
dd_Piz-t_F	ATCAGTCAGCTCGCAATGTG	144	ddPCR - *Piz-t* copy number determination
dd_Piz-t_R	TCTTGCCTAAACCACCCATC		

Table 3.4 Results of the amplification of 106 *M. oryzae* isolates with the 24 pairs of primers

Collection year	Country	Host	Isolate	FDWSRU no.	Pot2a	MoT3	PWL1	PWL2	PWL3	PWL4	AVR-Pia	AVR-Pib	AVR-Pii	AVR-Pik	AVR-Pikm	AVR-pita1	AVR-pita2	AVR-pita3	AVR-Piz-t	AVR-P9	AVR-Pi54	AVR1-CO39	ACE1	AVR-Rmg8	BAS1	BAS2	BAS3	BAS4
1986 - 1989	Brazil	*T. aestivum*	T-3	BR86Ta003	P	N	N	N	N	P	N	P	N	N	N	N	N	P	P	P	P	P	P	P	N	P	P	P
	Brazil		T-50	BR86Ta050	P	P	N	N	N	P	N	P	N	N	N	N	N	P+I	P	P	P	P	P	P	N	P	P	P
	Brazil	*T. aestivum*	T-2	BR87Ta002	P	P	N	N	N	P	N	P	N	N	N	N	N	P+I	P	P	P	P	P	P	N	P	P	P
	Brazil	*T. aestivum*	T-4	BR88Ta004	P	P	N	N	N	P	N	P	N	N	N	N	N	P+I	P	P	P	P	P	P	N	P	P	P
	Brazil	*T. aestivum*	T-5	BR88Ta005	P	N	N	N	N	P	N	P	N	N	N	N	N	P+I	P	P	P	P	P	P	N	P	P	P
	Brazil	*T. aestivum*	T-6	BR88Ta006	P	P	N	N	N	P	N	P	N	N	N	N	N	P+I	P	P	P	P	P	P	N	P	P	P
	Brazil	*T. aestivum*	T-7	BR88Ta007	P	P	N	N	N	P	N	P	N	N	N	N	N	P+I	P	P	P	P	P	P	N	P	P	P
	Brazil	*T. aestivum*	T-9	BR88Ta009	P	P	N	N	N	P	N	P	N	N	N	N	N	P+I	P	P	P	P	P	P	N	P	P	P
	Brazil	*T. aestivum*	T-12	BR88Ta012	P	N	N	N	N	P	N	P	N	N	N	N	N	P+I	P	P	P	P	P	P	N	P	P	P
	Brazil	*T. aestivum*	T-15	BR88Ta015	P	P	N	N	N	P	N	P	N	N	N	N	N	P+I	P	P	P	P	P	P	N	P	P	P
	Brazil	*T. aestivum*	T-17	BR88Ta017	P	P	N	N	N	P	N	P	N	N	N	N	N	N	P	P	P	P	P	P	N	P	P	P
	Brazil	*T. aestivum*	T-21	BR88Ta021	P	P	N	P	N	P	N	P	N	N	P+HB	N	N	P+I	P	P	P	P	P	P	P	P	P	P
	Brazil	*T. aestivum*	T-22	BR88Ta022	P	P	N	N	N	P	N	P	N	N	N	N	N	P+I	P	P	P	P	P	P	N	P	P	P
	Brazil	*T. aestivum*	T-25	BR88Ta025	P	P	N	N	N	P	N	P	N	N	N	N	N	P+I	P	P	P	P	P	P	N	P	P	P
	Brazil	*T. aestivum*	T-34	BR89Ta034	P	P	N	N	N	P	N	P	N	N	N	N	N	P+I	P	P	P	P	P	P	N	P	P	P
	Brazil	*T. aestivum*	T-37	BR89Ta037	P	P	N	N	N	P	N	P	N	N	N	N	N	P+I	P	P	P	P	P	P	N	P	P	P
	Brazil	*T. aestivum*	T-42	BR89Ta042	P	P	N	N	N	P	N	P	N	N	N	N	N	P+I	P	P	P	V	P	P	N	P	P	P
2005-2008	Brazil	*T. aestivum*	Py 5003	BR05Ta001	P	P	N	N	N	P	N	P	N	N	P+HB	N	N	P+I	P	P	P	P	P	P	N	P	P	P
	Brazil	*T. aestivum*	Py 6047	BR06Ta412	P	P	N	N	N	P	N	P	N	N	N	N	N	P+I	P	P	P	P	P	P	N	P	P	P
	Brazil	*T. aestivum*	Py 25.1	BR07Ta030	P	P	N	P	N	P	N	P	N	N	P+HB	N	N	P+I	P	P	P	P	P	P	N	P	P	P
	Brazil	*T. aestivum*	Py 35.3	BR07Ta051	P	P	N	N	N	P	N	P	N	N	N	N	N	P+I	P	P	P	P	P	P	N	P	P	P
	Brazil	*T. aestivum*	Py 86.1	BR08Ta416	P	N	N	N	P	N	N	P	N	N	P+HB	N	N	P+I	P	P	P	P	P	P	N	P	P	P
2012	Brazil	*T. aestivum*	Py 12.1.013	BR12Ta395	P	P	N	N	N	P	N	P	N	N	P+HB	N	N	P	P	P	P	P	P	P	N	P	P	P
	Brazil	*T. aestivum*	Py 12.1.014	BR12Ta396	P	P	N	P	N	P	N	P	N	N	P+HB	N	N	P	P	P	P	P	P	P	N	P	P	N
	Brazil	*T. aestivum*	Py 12.1.062	BR12Ta397	P	P	N	N	N	P	N	P	P	N	P+HB	N	N	P	P	P	P	P	P	P	N	P	P	P
	Brazil	*T. aestivum*	Py 12.1.063	BR12Ta398	P	P	N	N	N	P	N	P	N	N	P+HB	N	N	P	P	P	P	P	P	P	N	P	P	P
	Brazil	*T. aestivum*	Py 12.1.065	BR12Ta399	P	P	N	N	N	P	N	P	N	N	P+HB	N	N	P	P	P	P	P	P	P	N	P	P	P
	Brazil	*T. aestivum*	Py 12.1.147	BR12Ta401	P	P	N	N	N	P	N	P	N	N	P+HB	N	N	N	P	P	P	P	P	P	N	P	P	P
	Brazil	*T. aestivum*	Py 12.1.206	BR12Ta402	P	P	N	N	N	P	N	P	N	N	P+HB	N	N	N	P	P	P	V	P	P	N	P	P	P
	Brazil	*T. aestivum*	Py 12.1.207	BR12Ta408	P	P	N	N	N	P	N	P	N	N	P+HB	N	N	N	P	P	P	P	P	P	N	P	P	P
	Brazil	*T. aestivum*	16-Mot-01	BR16TA145	P	P	N	P	N	P	N	P	N	N	N	N	N	P+I	P	P	P	V	P	P	P	P	P	P
	Brazil	*T. aestivum*	16-Mot-02	BR16TA146	P	P	N	P	N	P	N	P	N	N	P+HB	N	N	P+I	P	P	P	V	P	N	P	P	P	P
	Brazil	*T. aestivum*	16-Mot-04	BR16TA147	P	P	N	P	N	P	N	P	N	N	P+HB	N	N	P+I	P	P	P	V	P	P	P	P	P	P
	Brazil	*L. multiflorum*	16-MoL-05	BR16LA148	P	N	N	P	N	N	N	N	N	N	P+HB	N	N	P+I	P	P	P	P	P	P	P	P	N	N
	Brazil	*T. aestivum*	16-Mot-08	BR16TA150	P	N	N	P	N	P	N	N	N	N	P+HB	N	N	P+I	P	P	P	P	P	P	P	P	P	N
	Brazil	*T. aestivum*	16-Mot-09	BR16TA151	P	N	N	P	P	P	N	N	N	N	P+HB	N	N	P	P	P	P	P	N	P	P	P	P	P
	Brazil	*T. aestivum*	16-Mot-10	BR16TA152	P	N	N	P	P	N	N	N	N	N	P+HB	N	N	P+I	P	P	P	P	P	P	P	P	P	N
	Brazil	*T. aestivum*	16-Mot-11	BR16TA153	P	N	N	P	P	N	N	N	N	N	P+HB	N	N	P+I	P	P	P	P	P	P	P	P	P	P
	Brazil	*T. aestivum*	16-Mot-12	BR16TA154	P	N	N	P	P	N	N	N	N	N	P+HB	N	N	P+I	P	P	P	N	P	P	P	P	P	P
	Brazil	*L. multiflorum*	16-MoL-14	BR16LA156	P	N	N	P	P	N	N	N	N	N	N	N	N	P+I	P	P	P	P	P	P	P	P	P	P
2016	Brazil	*T. aestivum*	16-Mot-15	BR16TA157	P	N	N	P	P	N	N	N	N	N	P+HB	N	N	P+I	P	P	P	P	P	P	P	P	P	P
	Brazil	*T. aestivum*	16-Mot-17	BR16TA158	P	N	N	N	N	P	N	N	N	N	P+HB	N	N	P+I	P	P	P	P	P	P	N	P	P	P
	Brazil	*T. aestivum*	16-Mot-19	BR16TA159	P	N	N	P	P	N	N	N	N	N	P+HB	N	N	P+I	P	P	P	P	P	P	P	P	P	P
	Brazil	*T. aestivum*	16-Mot-22	BR16TA160	P	N	N	N	P	N	N	N	N	N	P+HB	N	N	P+I	P	P	P	P	P	P	P	P	P	P
	Brazil	*T. aestivum*	16-Mot-23	BR16TA161	P	N	N	P	P	N	N	N	N	N	P+HB	N	N	P+I	P	P	P	P	P	P	P	P	P	P
	Brazil	*T. aestivum*	16-Mot-27	BR16TA162	P	N	N	P	P	N	N	N	N	N	P+HB	N	N	P+I	P	P	P	P	P	P	P	P	P	P
	Brazil	*T. aestivum*	16-Mot-28	BR16TA163	P	N	N	P	P	N	N	N	N	N	P+HB	N	N	P+I	P	P	P	P	P	P	P	P	P	P
	Brazil	*T. aestivum*	16-Mot-30	BR16TA164	P	P	N	P	N	P	N	N	N	N	P+HB	N	N	P+I	P	P	P	P	P	P	P	P	P	P
	Brazil	*T. aestivum*	16-Mot-31	BR16TA165	P	N	N	P	P	N	N	N	N	N	P+HB	N	N	P+I	P	P	P	P	P	P	P	P	P	P
	Brazil	*T. aestivum*	16-Mot-32	BR16TA166	P	N	N	P	P	N	N	N	N	N	P+HB	N	N	P+I	P	P	P	P	P	P	P	P	P	P
	Brazil	*T. aestivum*	16-Mot-33	BR16TA167	P	N	N	P	P	N	N	N	N	N	P+HB	N	N	P+I	P	P	P	P	P	P	P	P	P	P
	Brazil	*T. aestivum*	16-MoT-34		P	N	N	P	P	N	N	N	N	N	P+HB	N	N	P+I	P	P	P	P	P	P	P	P	P	P
	Brazil	*T. aestivum*	16-Mot-35	BR16TA168	P	N	N	P	P	N	N	N	N	N	P+HB	N	N	P+I	P	P	P	P	P	P	P	P	P	P
	Brazil	*T. aestivum*	16-Mot-36	BR16TA169	P	N	N	N	P	N	N	N	N	N	P+HB	N	N	P+I	P	P	P	P	P	P	N	P	P	P
	Brazil	*T. aestivum*	17-Mot-37	BR17TA170	P	P	N	P	N	P	N	P	N	N	N	N	N	P+I	P	P	P	V	P	N	P	P	P	P
	Brazil	*T. aestivum*	17-Mot-38	BR17TA171	P	P	N	N	N	P	N	P	N	N	P+HB	N	N	P+I	P	P	P	P	P	P	P	P	P	P
	Brazil	*T. aestivum*	17-Mot-39	BR17TA172	P	P	N	P	N	P	N	P	N	N	N	N	N	P+I	P	P	P	P	P	P	P	P	P	P
	Brazil	*D. horizontalis*	17-MoD-40	BR17DG173	P	P	N	N	N	P	N	P	N	N	N	N	N	P+I	P	P	P	P	P	P	N	P	P	P
2017	Brazil	*T. aestivum*	17-Mot-41	BR17TA174	P	P	N	N	N	P	N	P	N	N	P+HB	N	N	P+I	P	P	P	P	P	P	N	P	P	P
	Brazil	*T. aestivum*	17-Mot-42	BR17TA175	P	P	N	N	N	P	N	P	N	N	N	N	N	P+I	P	P	P	P	P	P	N	P	P	P
	Brazil	*T. aestivum*	17-Mot-43	BR17TA176	P	P	N	P	N	P	N	P	N	N	P+HB	N	N	P+I	P	P	P	V	P	N	P	N	P	P
	Brazil	*T. aestivum*	17-Mot-45	BR17TA177	P	P	N	P	N	P	N	P	N	N	N	N	N	P+I	P	P	P	P	P	P	P	P	P	P
	Brazil	*T. aestivum*	17-Mot-46	BR17TA178	P	P	N	P	N	P	N	P	N	N	N	N	N	P+I	P	P	P	P	P	P	P	P	P	P
	Brazil	*T. aestivum*	17-MoT-47	BR17TA179	P	P	N	P	N	P	N	P	N	N	P+HB	N	N	P+I	P	P	P	V	P	N	P	P	P	P

Collection year	Country	Host	Isolate	FDWSRU no.	Pot2a	MoT3	PWL1	PWL2	PWL3	PWL4	AVR-Pia	AVR-Pib	AVR-Pii	AVR-Pik	AVR-Pikm	AVR-pita1	AVR-pita2	AVR-pita3	AVR-Piz-t	AVR-Pi9	AVR-Pi54	AVR1-CO39	ACE1	AVR-Rmg8	BAS1	BAS2	BAS3	BAS4
2011	Bolivia	*T. aestivum*	B-1		P	P	N	N	N	P	N	P	N	N	P+HB	N	N	P+I	P	P	P	V	P	P	N	P	P	P
	Bolivia	*T. aestivum*	B-2	BO11Ta001	P	P	N	N	N	P	N	P	N	N	P+HB	N	N	P+I	P	P	P	V	P	P	N	P	P	P
	Bolivia	*T. aestivum*	B-4	BO11Ta003	P	P	N	N	N	P	N	P	N	N	P+HB	N	N	P+I	P	P	P	V	P	P	N	P	P	P
	Bolivia	*T. aestivum*	B-8	BO11Ta007	P	P	N	N	N	P	N	P	N	N	P+HB	N	N	P+I	P	P	P	P	P	P	N	P	P	P
	Bolivia	*T. aestivum*	B-17	BO11Ta009	P	P	N	N	N	P	N	P	N	N	P+HB	N	N	P+I	P	P	P	N	P	P	N	P	P	P
	Bolivia	*T. aestivum*	B-30	BO11Ta010	P	P	N	N	N	P	N	P	N	N	P+HB	N	N	P+I	P	P	P	P	P	P	N	P	P	P
2012	Bolivia	*T. aestivum*	B-52	BO12Ta019	P	P	N	N	N	P	N	P	N	N	P+HB	N	N	P+I	P	P	P	P	P	P	N	P	P	P
	Bolivia	*T. aestivum*	B-53	BO12Ta020	P	N	N	N	N	P	N	N	P	N	P+HB	N	N	P+I	P	P	P	P	P	P	N	P	P	P
	Bolivia	*T. aestivum*	B-59	BO12Ta026	P	P	N	N	N	P	N	P	N	N	P+HB	N	N	P+I	P	P	P	N	P	P	N	P	P	P
	Bolivia	*T. aestivum*	B-60	BO12Ta027	P	P	N	N	N	P	N	P	N	N	P+HB	N	N	P+I	P	P	P	P	P	P	N	P	P	P
	Bolivia	*T. aestivum*	B-61	BO12Ta028	P	N	N	N	N	P	N	N	P	N	P+HB	N	N	P+I	P	P	P	P	P	P	N	P	P	P
	Bolivia	*T. aestivum*	BRI-B71	BO12Ta029	P	P	N	P	N	P	N	P	N	N	P+HB	N	N	P	P	P	P	N	P	P	P	P	P	P
	Bolivia	*T. aestivum*	BRI-B70	BO12Ta300	P	P	N	N	N	P	N	P	N	N	P+HB	N	N	P+I	P	P	P	P	P	P	N	P	P	P
	Bolivia	*T. aestivum*	BRI-B72	BO12Ta301	P	P	N	P	N	P	N	P	N	N	P+HB	N	N	P+I	P	P	P	V	P	P	P	P	P	P
	Bolivia	*T. aestivum*	BRI-B73	BO12Ta302	P	P	N	P	N	P	N	P	N	N	P+HB	N	N	P+I	P	P	P	P	P	P	P	P	P	P
2014	Bolivia	*T. aestivum*	BRI-B104	BO14Ta311	P	P	N	N	N	N	N	N	N	N	N	N	N	N	N	N	P	N	N	N	N	N	P	N
	Bolivia	*T. aestivum*	BRI-B105	BO14Ta312	P	P	N	N	N	N	N	N	N	N	N	N	N	N	N	N	P	N	N	N	N	N	P	N
	Bolivia	*T. aestivum*	BRI-B107	BO14Ta314	P	N	N	P	N	N	P	N	N	N	N	N	P	N	N	N	N	N	N	N	N	N	N	P
	Bolivia	*T. aestivum*	BRI-B112	BO14Ta319	P	P	N	N	N	P	N	P	N	N	P+HB	N	N	N	P	P	P	P	P	P	N	P	P	P
	Bolivia	*T. aestivum*	BRI-B113	BO14Ta320	P	P	N	N	N	NA	N	P	N	N	N	N	N	N	P	NA	NA	N	N	NA	N	NA	P	NA
	Bolivia	*T. aestivum*	BRI-B117	BO14Ta324	P	P	N	P	N	P	N	P	N	N	P+HB	N	N	P	P	P	N	N	P	N	P	P	P	P
	Bolivia	*T. aestivum*	BRI-B121	BO14Ta328	P	P	N	P	N	P	N	P	N	N	P+HB	N	N	P	P	P	P	N	P	N	P	P	P	P
	Bolivia	*T. aestivum*	BRI-B122	BO14Ta329	P	P	N	P	N	P	N	P	N	N	P+HB	N	N	P	P	P	P	N	P	N	P	P	P	P
	Bolivia	*T. aestivum*	BRI-B124	BO14Ta331	P	P	N	P	N	P	N	P	N	N	P+HB	N	N	P	P	P	P	N	P	N	P	P	P	P
2017	Bolivia	*T. aestivum*	BO17Ta021		P	P	N	P	N	P	N	P	N	N	P+HB	N	N	P	P	P	P	P	P	N	P	P	P	P
2012	Paraguay	*T. durum*	P-3	PA12Td039	P	P	N	P	N	P	N	P	N	N	P+HB	N	N	P+I	P	P	P	N	P	P	P	P	P	P
	Paraguay	*T. aestivum*	P-5	PA12Ta044	P	P	N	N	N	P	N	P	N	N	P+HB	N	N	N	P	P	P	N	P	P	N	P	P	P
	Paraguay	*T. aestivum*	P-6	PA12Ta045	P	P	N	N	N	P	N	P	N	N	P+HB	N	N	N	P	P	P	P	P	P	N	P	P	P
	Paraguay	*T. aestivum*	P-8	PA12Ta047	P	P	N	N	N	P	N	P	N	N	P+HB	N	N	N	P	P	P	P	P	P	N	P	P	P
	Paraguay	*T. aestivum*	P-9	PA12Ta048	P	P	N	N	N	P	N	P	N	N	P+HB	N	N	N	P	P	P	P	P	P	N	P	P	P
	Paraguay	*T. aestivum*	P-10	PA12Ta049	P	P	N	N	N	P	N	P	N	N	P+HB	N	N	N	P	P	P	P	P	P	N	P	P	P
	Paraguay	*T. aestivum*	P-11	PA12Ta050	P	P	N	N	N	P	N	P	N	N	P+HB	N	N	N	P	P	P	P	P	P	N	P	P	P
	Paraguay	*T. aestivum*	P-12	PA12Ta051	P	P	N	N	N	P	N	P	N	N	P+HB	N	N	N	P	P	P	P	P	P	N	P	P	P
	Paraguay	*T. aestivum*	P-14	PA12Ta053	P	P	N	N	N	P	N	P	N	N	P+HB	N	N	N	P	P	P	P	P	P	N	P	P	P
	Paraguay	*T. aestivum*	P-15	PA12Ta054	P	P	N	P	N	P	N	P	N	N	P+HB	N	N	P	P	P	P	N	P	P	P	P	P	P
	Paraguay	*T. aestivum*	P-16	PA12Ta055	P	P	N	P	N	P	N	P	N	N	P+HB	N	N	P	P	P	P	N	P	P	P	P	P	P
	Paraguay	*T. aestivum*	P-17	PA12Ta056	P	P	N	P	N	P	N	P	N	N	P+HB	N	N	P	P	P	P	N	N	P	P	P	P	P
	Paraguay	*T. aestivum*	P-20	PA12Ta059	P	P	N	N	N	P	N	P	N	N	P+HB	N	N	N	P	P	P	P	P	P	N	P	P	P
2014	Paraguay	*T. aestivum*	P-38	PA14TA04	P	P	N	N	N	P	N	P	N	N	P+HB	N	N	N	P	P	P	P	P	P	N	P	P	P
	Paraguay	*T. aestivum*	P-40	PA18TA01	P	P	N	P	N	P	N	P	N	N	P+HB	N	N	P+I	P	P	P	P	P	P	P	P	P	P
2011	USA	*T. aestivum*	KY-5506		P	N	N	P	N	P	N	N	N	N	P+HB	N	N	P+I	P	P	P	P	P	P	P	P	P	P

P	Positive amplification
N	No amplification
P+I	Positive amplification, PCR product with different size, sequence showed a 74 bp insertion - no high homology with another seq. in the GeneBank
P+HB	Positive amplification, PCR product with a different size, sequence with a high background
V	Vague result, weak band in the gel, sequence with a high background.
NA	Not Analized

Table 3.5 Frequency of occurrence of the markers and genes in MoT isolates collected in different years and countries.

Only isolates from *Triticum* were considered, and the isolate KY5506 was excluded from this analysis.

Marker/Gene	Year Collection							Country		
	1986-1989[1]	2005-2008[2]	2011[3]	2012[4]	2014[5]	2016[6]	2017[7]	Brazil	Bolivia	Paraguay
Pot2a	100	100	100	100	100	100	100	100	100	100
MoT3	82.35	80	100	93.55	90.91	18.18	100	64.52	88	100
PWL1	0	0	0	0	0	0	0	0	0	0
PWL2	5.88	20	0	29.03	54.55	90.91	90	51.61	36.00	33.33
PWL3	0	20	0	0	0	72.73	0	27.42	0	0
PWL4	100	80	100	100	70	31.82	100	74.19	87.50	100
AVR-Pia	0	0	0	0	9.09	0	0	0	4	0
AVR-Pib	100	80	100	93.55	72.73	18.18	100	69.35	80	100
AVR-Pii	0	0	0	9.68	0	0	0	1.61	8	0
AVR-Pik	0	0	0	0	0	0	0	0	0	0
AVR-Pikm	5.88	60	100	100	63.64	95.45	50	61.29	84	100
AVR-pita1	0	0	0	0	0	0	0	0	0	0
AVR-pita2	0	0	0	0	9.09	0	0	0	4	0
AVR-pita3	94.12	100	100	61.29	45.45	100	100	93.55	80	33.33
AVR-Piz-t	100	100	100	100	72.73	100	100	100	88	100
AVR-Pi9	100	100	100	100	70	100	100	100	87.50	100
AVR-Pi54	100	100	100	100	80	95.45	100	98.39	91.67	100
AVR1-CO39	100	80	33.33	70.97	27.27	90.91	70	88.71	40	66.67
ACE1	100	100	100	96.77	63.64	95.45	100	98.39	84	93.33
AVR-Rmg8	100	100	100	96.77	30	90.91	50	88.71	66.67	100
BAS1	5.88	20	0	29.03	45.45	90.91	90	51.61	32	33.33
BAS2	100	100	100	100	70	100	90	98.39	87.50	100
BAS3	100	100	100	100	90.91	100	100	100	96	100
BAS4	100	100	100	96.77	80	90.91	100	95.16	91.67	100

[1]17 Brazilian isol.; [2]5 Brazilian isol.; [3]6 Bolivian isol.; [4]9 Brazilian, 9 Bolivian, and 13 Paraguayan isol.; [5]9 Bolivian and 2 Paraguayan isol.; [6]22 Brazilian isol.; [7]9 Brazilian and 1 Bolivian isol.

Chapter 4 - CRISPR/Cas9-mediated editing of the wheat endogenous genes *eIF(iso)4E-2* and *eIF4G* to reduce susceptibility to *Wheat streak mosaic virus* (WSMV)

Abstract

Wheat streak mosaic virus (WSMV), a member of the family *Potyvridae*, infects several cereals causing significant losses. In the Great Plains in the U. S., it can be found co-infecting wheat with two other viruses, *Triticum mosaic virus* and *High Plains wheat mosaic virus*, causing the wheat streak mosaic complex (WSM) disease. The incidence and severity of this disease varies from year to year, but in the last outbreak in 2017 in Kansas, it caused a yield loss of 5.6%, valued at $76.8 million. Members of the *Potyviridae* family use components of the eukaryotic translation initiation complex eIF4F or eIF(iso)4F, which include the factors eIF4E/eIF4G or eIF(iso)4E/eIF(iso)4G, respectively, to complete their infection cycle. Recessive resistance to the *Potyviridae* family has been identified in some plant species, and this has been frequently linked with natural allelic variants that prevent interaction between the virus and some component of the complex eIF4F/eIF(iso)4F. In a previous study, the silencing of two wheat factors *eIF(iso)4E-2* and *eIF4G* mediated by RNA interference (RNAi), resulted in a reduction in WSMV infection. The objective of this study was to implement a CRISPR/Cas9 system to induce mutations in the host factors *eIF(iso)4E-2* and *eIF4G* with the aim of generating transgene-free edited wheat plants with resistance to WSMV. Embryogenic calli from the susceptible cultivar 'Bobwhite' were independently co-transformed via biolistics with DNA plasmids carrying the wheat-optimized *Cas9* gene, sgRNAs targeting different sites in the *eIF(iso)4E-2* and *eIF4G*, and the herbicide resistance gene *bar*. A total of 102 T_0 transgenic

plants (positive for *Cas9* and sgRNA) were recovered, and four T_0 plants with CRISPR/Cas9-induced mutations in the target sites were recovered. The plant 4385 had mutations in the six alleles of *eIF(iso)4E-2*, whereas the plants 3380, 4129 and 5697 had mutations in one or two alleles of *eIF4G*. In the analyses of the progeny of plants 4385 and 5697, T_1 and T_2 plants with mutations in homozygous state were identified, as well as transgene-free edited plants. Plants with mutations in the six *eIF4G* alleles were not recovered, suggesting that this gene is essential for normal wheat development. Expression analysis of the different translation initiation factor genes *eIF4E*, *eIF(iso)4E-2*, *nCBP* (new cap-binding protein), *eIF4G* and *eIF(iso)4G-1/-2* in CRISPR/Cas9 edited plants showed a decrease in the expression in the targeted gene but not in the other isoforms. T_2 plants from the CRISPR/Cas9-edited lines 4385.A.5, 4385.B.12, 4385.B.16, 5697.A.5, 5697.A.12, and 5697.B.9/B.12, with different mutation profiles in the targeted genes, and control plants Bobwhite wild-type and 'RonL' were challenged with WSMV isolate 'Sidney 81'. Resistance to WSMV infection was not observed in any of the edited lines, typical symptoms were observed in the leaves a month after the first inoculation and virus accumulation in leaf tissue was confirmed by quantitative PCR (qPCR). A slight reduction in virus titer was detected in lines 5697.A.12, and 5697.B.12 when compared to Bobwhite_WT. The results obtained open many questions about the role played by eIF(iso)4E-2 and eIF4G in WSMV infection, and more studies should be conducted to try to identify the factors that interact with WSMV. CRISPR-mediated genome editing approaches have great potential, and this study demonstrated that CRISPR/Cas9 was useful to induce loss-of-function of the target genes in wheat, but more information is needed to select the target genes, and the creation of new alleles that can confer broad virus resistance through the use of variants of CRISPR methodology should be considered in the future.

Introduction

Wheat streak mosaic virus (WSMV) infects several cereals, including wheat, oat, barley, maize, millet, and many other grasses (Chalupníková et al., 2017). Wheat streak mosaic virus was first reported in Nebraska, in the U.S. Great Plains, in 1922 (McKinney,1937), and currently is widely distributed in the wheat-growing regions around the globe (Hadi et al., 2011). In the U.S. Great Plains, WSMV can be found in a complex (wheat streak mosaic complex -WSM) with other wheat viruses, *Triticum mosaic virus* (TriMV), and *High Plains wheat mosaic virus* (HPWMoV) (Burrows et al., 2009), although WSMV is the most prevalent in winter wheat and it can be detected as single infection (Byamukama et al., 2013, Byamukama et al., 2016). The three viruses are vectored by the wheat curl mite (WCM), *Aceria tosichella* Keifer (Slyknuis, 1955; Seifers et al., 1997; Seifers et al., 2009). Yield loss due to WSM is variable year by year, in 2017 it ranked as the second most important wheat disease in Kansas, causing a loss of 19.3 million bushels (equivalent to 5.6% estimated loss statewide) (Hollandbeck et al., 2017).

WSMV is a non-enveloped, flexible, filamentous and rod-shaped virus with a monopartite, positive-sense, single-stranded RNA (ssRNA+) genome (~ 9.3 kb) (Sing et al. 2018). WSMV genome encodes a single polyprotein of 3035 amino acids with the typical organization of the *Potyviridae* family, and it is the type member of the genus *Tritimovirus* (Stenger et al., 1998). The polyprotein has cleavage sites, resulting in 10 mature proteins (PI, HC-Pro, P3, 6K1, CI, 6K2, VPg, NIa, NIb, CP) (Stenger et al., 1998). The RNA has the VPg (viral protein genome-linked proteinase) at the 5'-terminus, and a poly(A) tail at the 3'-terminus, serving as a viral RNA messenger (Singh et al., 2018). Due to the relatively small genomes of plant viruses, they do not encode all the proteins they need to complete their infection cycle and must rely on several host proteins (Safaçon, 2015).

WSM symptoms in leaves start as small chlorotic lines, which eventually elongate forming discontinuous yellow to pale green streaks parallel to the leaf veins (mosaic pattern). Large chlorotic areas could result from coalescence of the stripes in the most severe cases (Hadi et al., 2011). Stunting is another characteristic symptom in infected plants (Hadi et al., 2011). Virus infection reduces the photosynthetic capacity of the plant, reduces root biomass and water use efficiency, resulting in low tillering of plants, low production of spikes or poorly filled kernels with reduced grain test weight (Hadi et al., 2011; Price et al., 2010; Singh et al., 2018). Symptoms in plants co-infected with WSMV and TriMV are more severe, and viral particles accumulate at higher levels than in single infections (Tatineni et al., 2010). High yield loss is associated with infection at the early stage of wheat development, and severe affected winter wheat plants are the result of infections that occurred in the autumn. (Singh et al., 2018). Disease management relies on cultural practices, especially in the elimination of host plants like volunteer wheat, maize and other grasses, which remain green during the summer ("green bridges") and allow the WCM vector and virus to survive among wheat crops (Tatineni & Hein, 2018). Few sources of genetic resistance to WSMV are available, and only three genes have been identified. *Wsm1* was identified from the wheat grass *Thinopyrum intermedium* (Friebe et al., 1991) and it has been transferred to the wheat cultivar Mace (Graybosh et al., 2009). *Wsm2* was identified in the wheat germplasm line CO960293-2 (Haley et al., 2002) and it has been deployed into cultivars RonL (Seifers et al., 2006), Snowmass (Haley et al., 2011), Clara CL (Martin et al., 2014), Oakley CL (Zhang et al., 2015), and Joe (Zhang et al., 2016). *Wsm1* and *Wsm2* are temperature sensitive, and they are only effective against *WSMV* and *TriMV*, or *WSMV,* respectively, at or below 18°C (Haley et al., 2011; Seifers et al., 1995; Seifers et al., 2007; Tatineni et al., 2010). Tatineni et al. (2016) reported that resistance conferred by *Wsm1*

and *Wsm2* is associated with a temperature-dependent blockage of viral long-distance transport due to inability of viruses to enter the vasculature. *Wsm3* is derived from *T. intermedium* (Liu et al., 2011) and it was translocated into the line KS12WGGRC59 (Friebe et al., 2011). *Wsm3* confers resistance to TriMV and WSMV up to 21°C and 24°C, respectively (Friebe et al., 2011; Kumssa et al., 2017).

Genetic engineering for resistance to WSMV in wheat has been implemented before. These strategies have been based primarily on two of the main plant antiviral defense responses, RNA silencing and recessive inherited resistance. In RNA silencing, the plant DICER-like RNAases (DCLs) process double-strand RNA (dsRNA) fragments generated by virus during their replication or transcription, producing short interfering RNAs (siRNAs). siRNAs are loaded onto Argonaute (AGO) proteins to form the RNA-inducing silencing complex (RISC), which recognizes complementary RNA molecules and degrades them or inhibits their translation (Schmitt-Keichinger, 2019). This mechanism explains what was previously known as pathogen-derived resistance and later as RNA-mediated virus resistance (Lindbo & Dougherty, 2005). For example, Sivamani et al. (2000) transformed immature embryos of the wheat cv. 'Hi-Line' with the *NIb* (Nuclear Inclusion polymerase) gene of WSMV, and plants showed resistance, with mild virus symptoms and development of new asymptomatic tissue. Later, two groups showed that the expression of the *CP* (coat protein) gene of WSMV in transgenic plants also conferred resistance to virus infection (Li et al., 2005; Sivamani et al., 2002). Instead of using full-length constructs, Fahim et al. (2010) and Cruz et al. (2014) used hairpin constructs with portions of the *NIa* (Nuclear Inclusion protease) and *CP* genes, respectively, to induce RNAi. Both groups reported that transgenic plants were highly resistant to virus infection, and that this resistance was observed until advanced generations. Alternatively, Fahim et al. (2012) used an artificial miRNA

construct to target 5 different regions in the WSMV genome. They found different levels of resistance in wheat transgenic plants, from immunity to break-down of the resistance in subsequent generations. In a recent study, Tatineni et al. (2020) used a hairpin construct with fragments of the *NIb* genes from WSMV and TriMV to transform the wheat genotype CB037. Some of the recovered transgenic plants were resistant to either WSMV of TriMV, some were susceptible to both viruses, and some showed resistance to both viruses. Authors reported that this dual resistance was observed up to the T_4 generation.

In recessive resistance, a plant factor essential for the virus cycle can no longer be used by the virus due to a mutation, and this loss-of-susceptibility can be associated with any gene involved in the viral infection cycle, like translation, replication or movement (Schmitt-Keichinger, 2019). A crucial step in virus infection is the translation of their genome, but since most viruses do not have the 7-methyl guanosine cap structure at the 5' end of the RNA (5'-cap), they have evolved an array of mechanisms to translate their genome using the host machinery (Dreher & Miller, 2006), for example, viruses from the *Potyviridae* family use the VPg linked to the 5 'end of their RNA to recruit host translation initiation factors eIF4F, which include the proteins eIF4E (small cap-binding protein), eIF4G (large scaffold protein) and their isoforms (Dreher & Miller, 2006; Zhang et al., 2015). Most of the recessive resistance has been associated with translation, and most of the recessive resistance to the family *Potyviridae* has been linked with the factors eIF4 (Robaglia & Caranta, 2006; Schmitt-Keichinger, 2019). For example, the resistance to *Potato virus Y* (PVY) in two resistant cultivars of pepper was associated with natural mutations in the *eIF4E* gene (Ruffel et al., 2002). Likewise, genes related with lettuce resistance to *Lettuce mosaic virus* (LMV), with pea resistance to *Pea seed-borne mosaic virus* (PSbMV), and with tomato resistance to PVY and *Tobacco etch virus* (TEV), corresponded to

natural variations in the gene *eIF4E* (Gao et al., 2004; Nicaise et al., 2003; Ruffel et al., 2005). Regarding monocots, it was demonstrated that the barley genes *rym4* and *rym5*, which confer resistance to *Barley yellow mosaic virus* (BaYMV) and *Barley mild mosaic virus* (BaMMV) corresponded to allelic variants of the *eIF4E* gene (Kanyuka, et al., 2005; Stein et al., 2005). Biotechnology has been a valuable tool to engineer resistance to *Potyviridae*. The silencing (mediated by RNAi) or knock-out (mediated by CRISPR/Cas9) of the genes *eIF4E*, *eIF(iso)4E* or *nCBP* (novel cap-binding protein) conferred resistance to *Potyviridae* in several crops, including tomato (Mazier et al., 2011), melon (Rodríquez-Hernández et al., 2012), plum (Wang et al., 2013), cucumber (Chandrasekaran et al., 2016), and cassava (Gomez et al., 2019). Rupp et al. (2019) identified the wheat *eIF(iso)4E-2* as the homolog of the barley *rym4/5 eIF4E* gene. They used an RNAi hairpin to target *eIF(iso)4E-2* and *eIF4G*, and they found that both the *eIF(iso)4E-2*- and *eIF4G*- silenced lines were resistant to WSMV, TriMV and *Soil-borne wheat mosaic virus* (SbWMV), in addition the resistance was stable up to the T_5 generation and it was inherited to the progeny of crosses with the cv. 'Karl 92' (Rupp et al., 2019). Despite the promising results obtained from the silencing of the wheat endogenous genes *eIF(iso)4E-2* and *eIF4G*, the effectiveness of this resistance depends on the active expression of the transgene (hairpin construct), therefore the incorporation of this resistance to commercial materials has numerous limitations. The CRISPR-based genome editing is a valuable alternative to knockout wheat genes, since transgene-free plants carrying the desired modifications can be recovered (Kumar et al., 2019). The objective of this study was to implement the CRISPR/Cas9 technology to modify the wheat *eIF(iso)4E-2* and *eIF4G* genes. We hypothesize that wheat plants with *eIF(iso)4E-2*- and *eIF4G*- CRISPR/Cas9-edited genes should be resistant to WSMV.

Materials and Methods

Selection of Cas9 target sites

Sequences of the proteins eIF(iso)4E-2 and eIF4G (Uniport Q03389 and G5CEW6, respectively) were used as query in the tblastn program in EnsemblPlants (https://plants.ensembl.org/Triticum_aestivum/) against the bread wheat reference sequence IWFSC RefSeq v1.0. Scaffolds from the three genomes were aligned with the coding sequences reported in the NCBI (AAA34296 and AEQ49596) to identify introns/exons in the homeologous. The consensus sequences were used to identify potential genomic target sites (20 nucleotides plus a 5′-NGG-3′ protospacer adjacent motif - PAM) using the software CRISPRdirect (https://crispr.dbcls.jp/).

For each gene, four genomic target sites were selected based on these parameters: (i) the target sequence is present in the three genomes/homeologous (hit_20mer column in Table 4.1); (ii) the 20-nt sgRNA has no homology to potential off-target sites; (iii) the 12-nt sgRNA has homology to few potential off-target sites (column hit_12mer in Table 4.1); (iv) the potential off-target sites are in non-coding regions; and (v) the target site has a 35 to 80% GC content. In addition, some of the selected target sites were located inside the region used for the RNA interference experiments reported by Rupp et al. (2019).

Cloning target-specific oligos into a sgRNA scaffold vector

pTaU6_sgRNA vector was synthesized by GenScript (Piscataway, NJ) by cloning the sgRNA scaffold sequence (Shan et al., 2014) under the control of the *T. aestivum* U6 promoter, into the pUC57 vector. The vector had two *BbsI* restriction sites that allowed replacing a fragment of the plasmid with each of the 20-nt target specific sequences. Briefly, the pTaU6_sgRNA vector was digested with the restriction enzyme *Bbs*I (New England BioLabs,

Ipswich, MA), and the 5′ and 3′ends were dephosphorylated with Shrimp Alkaline Phosphatase rSAP (New England BioLabs, Ipswich, MA) according to the manufacture's protocol. The digested vector was purified using QIAquick® PCR purification kit (QIAGEN, Germantown, MD), adjusted to 50 ng/µL, and stored at -20°C until ligation. Forward and reverse target-specific oligos were synthesized by IDT (Integrated DNA Technologies, Coralville, Iowa). To facilitate the ligation of the oligos into the sgRNA scaffold vector in the proper orientation, CTTG and AAAC sequences were added to the 5′end of the forward and reverse oligos, respectively (Table 4.2); additionally a phosphate group at the 5' end of the forward and reverse oligos was incorporated to favor ligation. Oligos were annealed in a thermocycler by decreasing the temperature from 95°C to 25°C (-1°C min^{-1}), in a 20 µL reaction volume, with a final concentration of 4.5 µM of each oligo. Ligation was performed using 50 ng of digested vector, 3 µL of annealed oligos, and 3 units of T4 DNA ligase (Promega, Madison, WI), following manufacture's recommendations. *Escherichia coli* TOP10 chemically competent cells were transformed using 4 µL of ligation mix according with the standardized protocol. Colonies were evaluated by direct PCR, and plasmids from positive colonies were purified using the E.Z.N.A.® plasmid mini kit II (OMEGA, Norcross, GA) and sent to sequence to Genewiz (South Plainfield, NJ) to confirm the insertion of the target-specific oligos.

Biolistic transformation of wheat and identification of transgenic plants

Embryogenic calli from the spring wheat cultivar 'Bobwhite' were co-bombarded using the standardized particle inflow gun-mediated protocol described in Chapter 2 (Tian et al., 2019). Tungsten particles were prepared using 2 ng of each plasmid: (i) bar_pAHC20 (*bar* gene under control of the maize *Ubiquitin Ubi1* promoter and Nos terminator) (Christenson and Quail, 1996), (ii) pTawoCas9 (wheat-codon-optimized *Streptococcus pyrogenes* Cas9 cloned under

control of the *Ubi1* promoter and *Nos* terminator in pAH17), and (iii) each of the eight pTaU6_sgRNA vectors described before. A total of fifty-five independent bombardment experiments were completed. Regeneration of shots, rooting, and transfer of plantlets to soil was achieved as described before (Chapter 2). The presence of the transgenes in regenerated plantlets was tested by direct amplification with the KAPA3G Plant PCR kit (Kapa Biosystems, Indianapolis, IN). Crude extract was obtained by crushing a piece of leaf lamina (approximately 0.5 cm x 0.5 cm) with a micropipette tip in 100 µl of extraction buffer (50 mM Tris-HCl pH 8.0, 0.1 mM EDTA pH 8.0, 2% β-mercapto-ethanol). The tubes were preserved on ice, incubated at 95 °C for 5 minutes and put back on ice. The crude extract was diluted with double-distilled water at 1:10 ratio and used freshly in PCRs. Amplification of Cas9 and sgRNA was done in a duplex reaction with the primers TaCas9_FWD, TaCas9_REV, TaU6_FWD and sgRNA_REV (Table 4.3), using 1X KAPA PCR buffer (Kapa Biosystems, Indianapolis, IN), 1 mM $MgCl_2$, 0.3 µM each primer, 1 unit KAPA3G polymerase (Kapa Biosystems, Indianapolis, IN), 5 µl of crude extract diluted, in a final volume of 25 µl. Reactions were completed on a Multigene Gradient thermal cycler (TC9600G, Labnet, Edison, NJ) with 95 °C for 6 minutes, 40 cycles of 95 °C for 30 seconds, 62 °C for 30 seconds, and 72 °C for 45 seconds, and a final extension at 72 °C for 10 minutes. The *bar* gene was amplified using the primers UbiABF and BarABR (Table 4.3) following the conditions reported.

Detection of target-site mutations in transgenic plants

DNA was isolated from positive transgenic plants (tillers) using the DNeasy® Plant Mini Kit (QIAGEN, Germantown, MD). The presence of transgenes *Cas9*, *sgRNA* and *bar* was validated using genomic DNA by PCR. Possible mutations in target sites were analyzed by amplification and sequencing of regions flanking the target sites. Forward and reverse primers

for each flanking region were designed (Table 4.3) and used in 25 μL reactions containing 1X GoTaq® Flexi buffer (Promega, Madison, WI), 2.5 mM $MgCl_2$, 400 μM each dNTP, 0.4 μM each primer, 1 unit of GoTaq® Flexi DNA polymerase (Promega, Madison, WI), and 25 ng genomic DNA. All reactions were completed on a Multigene Gradient thermal cycler (TC9600G, Labnet, Edison, NJ) with initial denaturation at 94 °C for 5 minutes, 35 cycles of 94°C for 30 seconds, 60/62°C for 30 seconds (60°C for primers flanking sites is the *eIF(iso)4E-2* gene, and 62°C for primers flanking sites in the *eIF4G*), and 72 °C for 45 seconds, and a final extension at 72 °C for 10 minutes. PCR products were purified using the QIAquick® PCR purification kit (QIAGEN, Germantown, MD) and sent to sequencing to Genewiz (South Plainfield, NJ). Sequence data were analyzed using the Geneious R9 software v.9.1.8 (Biomatters Ltd). The presence of high background (double peaks) in the sequence chromatogram suggested mutation in the target site.

For those target sites with a restriction enzyme recognition site inside (Table 4.1), the presence of a mutation was additionally tested by PCR/RE analysis. The region flanking the target site was amplified by PCR according with the conditions described before, the PCR product was column purified and 1 μg was digested with the corresponding enzyme, following manufacture´s recommendations. The loss of the enzyme recognition site due to a mutation was detected by the presence of un-cleaved bands in agarose gels (1.5% agarose in 1X TAE electrophoresis buffer).

Characterization of mutations in T_0 plants

Plants whose sequence analysis of flanking regions showed double peaks in the chromatograms, suggesting the presence of mutations, were analyzed by sub-cloning and sequencing. The regions flanking the target site were amplified in 25 μL reactions using 1X Q5®

High-Fidelity master mix (New England BioLabs, Ipswich, MA), 0.5 μM each primer (Table 4.3), and 50 ng genomic DNA. Reactions were done using a Multigene Gradient thermal cycler (TC9600G, Labnet, Edison, NJ) with initial denaturation at 98°C for 30 seconds, 35 cycles of 98°C for 10 seconds, the corresponding annealing temperature for 20 seconds (66 °C for 4E2.30 site, 65 °C for 4G.523 site, 67 °C for 4G.2999 site, and 63 °C for 4G.3454 site), and 72 °C for 30 seconds, and a final extension at 72 °C for 7 minutes. The PCR products were cloned into the pCR™4Blunt-TOPO® vector (Invitrogen, Carksbad, CA) according to manufacturer's protocol, and 2 μl of the cloning reaction were used to transform One Shot™ TOP10 Chemically competent *E. coli* (Invitrogen, Carksbad, CA), following the manufacture´s protocol. Colonies were tested by PCR using the primers M13 forward and M13 reverse (Table 4.3), and DNA plasmid from positive colonies was purified using the E.Z.N.A.® plasmid mini kit II (OMEGA, Norcross, GA) and sent to sequence to Genewiz (South Plainfield, NJ). Sequence data were analyzed using the Geneious R9 software v.9.1.8 (Biomatters Ltd), and sequence alignments were made using Clustal Omega software (https://www.ebi.ac.uk/Tools/msa/clustalo/). The possible results of the mutations in the *eIF(iso)4E-2* and *eIF4G* genes were analyzed *in silico*. Reported sequences of the genes were modified according to the type of insertions or deletions (indels) detected and translated to proteins. Sequences of the modified proteins were aligned with the wild-type protein using Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/) to identify amino acid substitutions and premature stop codons.

Characterization of mutations in CRISPR/Cas9 edited progeny

To characterize mutations in the progeny T_1, T_2, and T_3, primers were designed to amplify specifically the region flanking the target site in each genome. The genotype of individual plants was determined based on PCR amplicon size (for large deletions), restriction

enzyme digestion, or sequencing. To characterize mutations in the target site 4E2.30 (first exon of the *eIF(iso)4E-2* gene, 30 bp from the start codon), genome specific forward primers were designed based on differences in the sequence of the 5'-UTR region (Table 4.3). Flanking regions were amplified with the forward genome-specific primers plus the 163_R3 reverse primer, in 25 µL reactions with 1X GoTaq® Flexi buffer (Promega, Madison, WI), 2.5 mM $MgCl_2$, 400 µM each dNTP, 0.4 µM each primer, 1 unit of GoTaq® Flexi DNA polymerase (Promega, Madison, WI), and 25 ng genomic DNA. Reactions were completed using a Multigene Gradient thermal cycler (TC9600G, Labnet, Edison, NJ) with initial denaturation at 94°C for 5 minutes, 35 cycles of 94 °C for 35 seconds, 60°C for 35 seconds, and 72°C for 45 seconds, with a final extension at 72°C for 10 minutes. Digestion of PCR products was done using 10 µL of reaction (un-purified), 2 µL of 10X CutSmart buffer (New England BioLabs, Ipswich, MA) and 10 units of the enzyme, in a final volume of 30 µL. Digestion was incubated at 37 °C for 90 to 120 minutes and products were analyzed in 1.3% agarose gels (in 1X TAE buffer). PCR products from the A genome (528 bp) were digested with the restriction enzymes *Drd*I and *Ahd*I. (New England BioLabs, Ipswich, MA), and PCR products from the B genome with a size of 421 bp were digested with the enzyme *Ddr*I (New England BioLabs, Ipswich, MA). PCR products from the D genome with a size of 415 bp were purified (QIAquick® PCR purification kit, QIAGEN, Germantown, MD) and sent for sequencing to Genewiz (South Plainfield, NJ).

Mutations in the target site 4G.523 (first exon of the *eIF4G* gene, 523 bp from the start codon) were evaluated by specific amplification of each genome with primers designed based on differences in the first intron of the gene (Table 4.3) and used together with the forward primer 523_F. Conditions for PCR and digestion were the same previously reported, but using 58°C

annealing temperature in PCRs. PCR products were digested with the enzyme of *Sfc*I or *Pst*I (New England BioLabs, Ipswich, MA).

Genome-specific primers were not designed to amplify the region flanking the target site 4G.2999 because this is located in a region with highly conserved sequence (third exon with 2816 bp long). Primers 2999_F and 2999_R were used to amplify the flanking region from the three genomes, PCR product was column purified (QIAquick® PCR purification kit, QIAGEN, Germantown, MD) and sent for sequencing to Genewiz (South Plainfield, NJ). Presence of mutations in individual plants were identified based on chromatograms.

Genome-specific reverse primers designed based on differences in the 3' end of the third exon were used with the 3454_F primer to detect mutations in the target site 4G.3454 (Table 4.3). PCRs were completed using the conditions reported above, using 54°C annealing temperature to amplify the B and D genome, and 60 °C to amplify the A genome. PCR amplicons from the B and D were digested with the enzyme *Btg*I (New England BioLabs, Ipswich, MA), as described previously. In addition, the region flanking the target site was amplified by PCR using the primers 3454_F and 3454_R, purified (QIAquick® PCR purification kit, QIAGEN, Germantown, MD) and sent for sequencing at Genewiz (South Plainfield, NJ). Analysis of chromatograms allowed the identification of mutations in the progeny.

Bioassays

The response of CRIPR/Cas9 edited lines to WSMV was tested in T_2 plants from two edited lines. Seeds from edited lines, together with control materials (Bobwhite_wild type and RonL) were sown in SureRoots® 50 containers (50 cells, 2 in x 2in x 5in, T.O. Plastics, Clearwater, MN). Seedlings were grown in chambers with 18°C/15°C day/night temperature, a 16 h photoperiod, with a light intensity of 450 $\mu E\ m^{-2}\ s^{-1}$, and 50 to 60% relative humidity. When

seedlings were in two-leaf stage (about 2 weeks after planting), both leaves were inoculated with WSMV isolate 'Sidney 81' ('Hays'). WSMV inoculum was prepared from fresh infected leaves, using a ratio of 1 g of infected tissue per 20 mL of 0.02 M phosphate buffer pH 7.2. The corresponding amount of infected tissue was macerated in ice-cooled buffer using a pre-cooled mortar and pestle. The mortar with the inoculum was kept on ice, and Carborundum powder was dispersed in a weight dish and placed on ice. A folded Kimwipe® (Kimberly Clark, Roswell, GA) was moistened with the inoculum and the excess of liquid was removed by pressing the wipe against the mortar wall. A small amount of carborundum power was taken with the moistened wipe and used to rub the surface (adaxial and abaxial) of the leaf lamina several times (about 8 times) until water-soaked spots were observed. For mock inoculated plants, the same procedure was carried out, but using only 0.02 M phosphate buffer pH 7.2. After inoculation, seedlings were returned to growth chambers. Eleven to twelve days after the first inoculation, plants were re-inoculated following the same procedure described before. Disease symptoms were scored 14 or 15 days after the second inoculation, using the WSMV rating scale proposed by Rupp (2015). Leaf tissue was collected from the youngest leaf of the main or second tiller (about 8 cm of the mid part of the lamina, ~ 100 mg) in 2.0 microcentrifuge tubes containing two 4.5 mm beads (BBs, Daisy®, Rogers, AR) and flash frozen in liquid nitrogen. Samples were stored at -80 °C and subsequently used to isolate total RNA. Specific details of each bioassay are presented below:

Bioassay 1: Seeds from the CRIPR/Cas9-edited lines 4385.A.5, 4385.B.5, and 4385.B.16 were tested along with the controls Bobwhite_WT and 'RonL'. These lines (T_2) carried mutations in the *eIF(iso)4E-2* gene, with homozygous mutations in each genome. Three trays were planted (5 x 10 cells) in a complete random design, with 10 repetitions of each material per

tray. When plants reached the two leaf-stage (15 days after seed germination), seedlings in two trays were inoculated with WSMV 'Sidney 81'('Hays') isolate, and seedlings from the third tray were mock inoculated with buffer. Eleven days after the first inoculation, the youngest leaf of the main tiller was re-inoculated with WSMV or mock inoculated. Disease symptoms were scored 15 days after the second inoculation, and tissue was collected 17 days after the second inoculation.

Bioassay 2: Seeds from the CRIPR/Cas9-edited lines 5697.B.9/B.12, 5697.A.5, and 5697.A.12 were evaluated against WSMV, together with the controls Bobwhite_WT and 'RonL'. These edited lines (T_2) had mutations in the *eIF4G* gene, with homozygous mutations in the A genome, in the D genome, or in the A and D genome, respectively. Due to the limited availability of seeds, seeds of both lines 5697.B.9 and B.12, with the same genotype, were used in the experiments. Two and a half trays were planted (5 x 10 cells) in a complete random design, with 10 repetitions of each material per complete tray. Seedlings in the two-leaf stage were WSMV or mock inoculated, following the protocol described before. Twelve days after the first inoculation the youngest leaf of the tillers was WSMV or mock re-inoculated. Fourteen days after the second inoculation, the disease symptoms were scored using the WSMV rating scale reported by Rupp (2015) and tissue (about 100 mg) from the middle lamella of the youngest leaf of the main tiller was collected.

Bioassay 3: Seeds from the CRIPR/Cas9-edited lines 4385.A.5.3 (T_3), 4385.B.12.1 (T_3), 4385.B.16.1 (T_3), 5697.A.5 (T_2), 5697.A.12 (T_2), 5697.B.12 (T_2), RNAi-silenced lines 1550.A.3.2.1 (T_4), 1673.A.3.2 (T_3) (Rupp et al., 2019), and controls Bobwhite_WT and Clara CL, were sown in trays. Four trays were sown, with 5 seeds of each line per tray, with the exception of the line 1673.A.3.2, because few seeds were available. Two weeks after planting,

seedlings from a tray were inoculated with WSMV isolate 'Sidney 81', seedlings from a second tray were inoculated with a WSMV isolate 'MHK', seedlings from a third tray were inoculated with buffer (mock-inoculated), and seedling for the four tray were not inoculated. Two weeks later, the youngest leaf of the main and secondary tiller (if present) were inoculated again. Two weeks after the second inoculation tissue was collected from all plants (about 8 cm foliar lamina of the youngest leaf of the main or second tiller) and stored at -80 °C.

Relative expression of the eIF4 factors and virus titer analysis

To evaluate the relative expression of the eIF4 factors and their isoforms in the CRISPR/Cas9 edited lines, two-step quantitative Real-Time PCR (RT-qPCR) assays were conducted. Protein sequences of the eIF4 factors (Accession P29557 for eIF4E-1, Q03389 for eIF(iso)4E-2, A3RCV9 for Novel cap-binding protein/eIF4E-3, G5CEW6 for eIF4G, Q03387 for eIF(iso)4G-1, and Q41583 for eIF(iso)4G-2) were used as query in the tblastn program (EnsemblPlants, https://plants.ensembl.org/Triticum_aestivum/), against the bread wheat reference sequence IWFSC RefSeq v1.0., to recover the gene sequences from the three genomes. Based on the consensus sequence from the alignments (Clustal Omega, https://www.ebi.ac.uk/Tools/msa/clustalo/), primers to amplify all the isoforms by qPCR were designed using Primer3 (http://primer3.ut.ee) (Table 4.3). Additionally, primers to amplify the *T. aestivum* housekeeping gene *actin* (Gene Bank accession Q5EWZ1) were also designed. To quantify the concentration of WSMV in inoculated plants (virus titer), primers were designed based on the sequence of WSMV strain 'Sidney 81' (accession AF057533) in the region encoding for NIa (Table 4.3). Total RNA was isolated from 100 mg of leaf tissue using the TRIzol® reagent (Invitrogen, Carlsban, CA), according to manufacturer's protocol. The RNA pellet was resuspended in 100 µl of RNase-free water, quantified by NanoDrop

(ThermoScientific, Waltham, MA) and stored at -80°C. Single-stranded cDNA was synthesized by reverse transcription from 1 μg of total RNA using the Reverse Transcription system (Promega, Madison, WI). cDNA was diluted (1:4 ratio) with RNase-free water and used for conventional PCRs (RT-PCR) and quantitative PCRs (RT-qPCR). Contamination of RNA with genomic DNA (gDNA) was tested by RT-PCR with the *Tubulin* primers (Tub-F and Tub-R), which generate an amplicon of 500 bp from gDNA, and a product of 408 bp from cDNA. For all qPCR primer pairs, the optimum annealing temperature (Ta) was established through a temperature gradient assay, using four annealing temperatures (56.6°C, 58.2°C, 60.1°C, and 61.7°C). The amplification efficiency of each pair of primers was assessed by building standard curves with 10-fold dilutions at the optimum annealing temperature. All qPCR reactions were done in a 20 μL final volume with 1X SsoAdvanced universal SYBR® Green supermix (Bio-Rad, Hercules, CA), 0.35 μM each primer, and 5 μL diluted cDNA (1:4). The reactions were completed in a Bio-Rad CFX96 Real-Time System (Bio-Rad, Hercules, CA) with polymerase activation/denaturation at 95°C for 30 sec, 40 cycles with denaturation at 95°C for 15 sec, annealing/extension temperature for 30 sec, and a melt curve analysis with 65°C to 95°C, with 0.5°C increments each 5 sec. The data was collected using the CFX Maestro™ Software (Bio-Rad, Hercules, CA). To determine the relative expression of the eIF4 genes in CRIPR/Cas9 edited lines, three biological replicates with three technical repetitions were run. Six biological replicates, with three technical repetitions, were used in viral titer assays. The relative expression was calculated considering the expression level of the reference gene (*actin*) and the expression level of the target gene (eIF4 or WSMV), according with the formula: Expression = $2^{\Delta Ct} = 2^{(Ct\ actin) - (Ct\ target)}$. The data were analyzed by one-way analysis of variance (ANOVA) using the GLM

procedure in the SAS software, Version 3.8, Enterprise Edition. Copyright© 2012-2018 SAS Institute Inc (Cary, NC).

Results

Recovery of transgenic plants

More than 8500 embryogenic calli from the cultivar 'Bobwhite' were used in bombardment experiments with the eight different pTaU6_sgRNA plasmids targeting *eIF(iso)4E-2* and *eIF4G*. A total of 527 regenerated plants were tested by direct PCR, 407 plants were negative for the three transgenes, *Cas9*, *sgRNA* and *bar*, and 120 plants were carrying at least one of the transgenes. The presence of the three transgenes was confirmed in 101 plants, and an additional plant was having *Cas9* and *sgRNA*, but not *bar*. The number of embryogenic calli bombarded with each of the 8 different sgRNAs and the number of positive transgenic plants carrying each sgRNA are presented in the Table 4.4. The average transformation efficiency for all co-bombardment experiments was 1.2%, ranging from 0.67% to 1.8%.

Identification and characterization of mutations in T_0 transgenic plants

Presence of mutations in the target site was evaluated in the 102 T_0 transgenic plants that carried *sgRNA* and *Cas9*. Amplification and sequencing of the regions flanking the target site allowed the identification of four T_0 transgenic plants whose chromatograms showed high background or double peaks, when compared with the Bobwhite_wild type chromatograms (Figure 4.1). Possible indels at the target site in some of the alleles of these putative mutant plants would result in a mixture of wild-type and mutated sequences that were visualized as overlapping peaks in the chromatograms. The plant 4385 showed putative mutation(s) at the target site 4E2.30 of the gene *eIF(iso)4E-2*, and plants 5697, 4121 and 3880 showed mutations at

the sites 4G.523, 4G.2999 and 4G.3454 of the gene *eIF4G*, respectively. Mutations in plants 3880 (tillers B, C, and D) and 5697 (tillers A, and B) were confirmed by digestion of the amplicons with the restriction enzymes *Btg*I and *Sfc*I (Figure 4.2). Undigested bands observed in the tillers of the mutant plants resulted from the loss of the enzyme recognition site due to the mutations induced by CRISPR/Cas9 in the target sites 4G.3454 and 4G.523.

Cloning and sequencing of the regions flanking the target sites confirmed targeted mutations in the four T_0 plants. Sequence analysis of the tillers A and B from 4385 revealed that all the six alleles contained mutations in the target site 4E2.30: in the A genome an allele showed a -1 bp deletion (C), and a second allele had a -2 bp deletion (CC); in the B genome an allele had a -1 bp deletion (C) and the second allele showed a -42 bp deletion; and in the D genome an allele had a +1 bp insertion (A), and the other allele had a -34 bp deletion. Since each allele in each genome carried a different type of mutation, mutations in each genome are trans-heterozygous (Figure 4.3 A). Sequence analysis of amplicons from the tillers 5697.A and 5697.B confirmed the presence of heterozygous mutations in the target site 4G.523 in the A and D genomes. In the A genome, an allele showed the wild-type sequence whereas the second allele had a +1 bp insertion (A), both alleles in the B genome had the wild-type sequence, and in the D genome an allele had a +1 bp insertion (T) and the second allele was wild-type (Figure 4.3 B). Sequence analysis of amplicons from 4129.A confirmed an heterozygous mutation in the target site 4G.2999 in the A genome, where an allele carried a -2 bp deletion (CT) and the second allele had the wild-type sequence. Alleles in the B and D genome were wild-type (Figure 4.3 C). A -1 bp deletion (C) was observed in the target site 4G.3454 in the plant 3880.B (Figure 4.3 D), nevertheless the low similarity between the polymorphisms (SNPs) observed in the sequences of the flanking region and the polymorphs of the reference sequences did not allow to identify in

which of the genomes the mutation was found, although a similar allele with the wild-type sequence was detected, suggesting that mutation was heterozygous. The editing frequencies for each of the sgRNAs used are presented in Table 4.4.

Based on the type of mutations identified in T_0 plants, an *in silico* analysis was done to establish the possible outcomes of these mutations in the proteins. "Edited" proteins were translated from the edited genes and compared to the wild-type proteins. Most of the indels (-1, -2 , -34 deletions, and +1 insertions) resulted in frameshift mutations, generating amino-acid substitutions and non-sense codons (Figure 4.4). For example, in the plant 4385, proteins resulting from the *eIF(iso)4E-2* gene with -1, -2 and +1 indels, would be shorter, (84, 74 and 81 amino-acids, respectively) than wild-type protein (209 a.a.), and these products would have amino-acid changes from the 16th residue (Figure 4.4 A). The protein generated from the *eIF(iso)4E-2* gene with the -34 deletion would be 74 a.a. length and have substitutions from the 10th a.a., whereas the protein produced from the gene with the -42 bp deletion would have a deletion of 14 a.a. at the N-terminus (Figure 4.4 A). Three different kind of mutations in the *eIF4G* gene were identified in three different plants. The protein generated in plants 5697 with a +1 insertion in the *eIF4G* gene would be 182 a.a. length, whereas the wild type protein is 1488 a.a. (Figure 4.4 B). In plants 4129 and 3880, proteins resulting from the *eIF4G* gene with -2 and -1 deletions would be short, with 1013 and 1205 a.a., and would have substitutions from the 1009th and 1163rd residues, respectively (Figure 4.4 C and D).

Characterization of mutations in the T_1 and T_2 progeny

Mutations in 47 T_1 plants derived from the mutant plant 4385 were characterized by specific amplification of each genome, digestion of the amplicons with restriction enzymes (*Ahd*I to identify -2 deletions in the A genome, and *Drd*I to identify -1 deletion in the A and B

genome), and sequencing (+1 insertion in the D genome) (Figure 4.5). Ten of characterized T_1 plants showed homozygous mutations in the three genomes (-1/-1 or -2/-2 in the A genome, -1/-1 or -42/-42 in the B genome, +1/+1 or -34/-34 in the D genome), and 20 T_1 transgene-free plants were identified. Mutation in 28 T_1 plants derived from the mutant 5697 were analyzed by specific amplification of each genome and digestion of the amplicons with the enzyme *Pst*I (Figure 4.6), only two plants carried homozygous mutations in the A and D genome (+1/+1 in the A genome, and +1/+1 in the D genome), and only one transgene-free plant (wild-type for the A and B genome, and +1/wt for the D genome) was found. Genome-specific primers were not designed for the region flanking the target site 4G.2999, nevertheless the sequence analysis of 28 T_1 plants derived from the mutant 4129 showed that 20 of these T_1 plants had mutations, including 2 transgene-free plants. Twenty-four T_1 plants derived from the mutant plant 3880 were analyzed by genome-specific amplification and restriction enzyme digestion. Several pair of primers were designed to amplify specifically the A genome, but multiple amplicons were produced in all the experiments. Amplification of the B and D genomes with the specific primers was successful, and digestion of the amplicons with the enzyme *Btg*I confirmed that alleles in these genomes were wild-type (complete digestion). The sequence analysis of the region flanking the target site (mixture of the three genomes) confirmed that all the T_1 plants evaluated were having a mutation. Results suggested that the mutation(s) in the target site 4G.3454 in the 3880 plants were in the A genome.

Genotype of the T_2 plants used in the bioassays were tested by genome-specific amplification and restriction enzyme digestion. All the T_2 plants evaluated showed the genotype corresponding to the T_1 homozygous parental.

Bioassays and relative quantification of translation factors

In the ***Bioassay 1***, T_2 plants from the *eIF(iso)4E-2* edited lines 4385.A.5 (A: -2/-2, B: -42/-42, D: -34/-34), 4385.B.12 (A: -1/-1, B: -1/-1, D: +1/+1) and 4385.B.16 (A: -1/-1, B: -42/-42, D: +1/+1); plus controls Bobwhite_WT and 'RonL' were challenged with WSMV isolate 'Sidney 81'. Seedlings from the same materials were also mock-inoculated with phosphate buffer. The relative expression of the target gene *eIF(iso)4E-2* and other isoforms was evaluated by RT-qPCR in mock- and WSMV-inoculated plants from the CRISPR/Cas9-edited lines and Bobwhite_WT. Results showed a strong reduction in the relative expression of the gene *eIF(iso)4E*-2 in the three edited lines, whereas significant differences in the relative expression of the other isoforms were not detected. This significant reduction in the expression of *eIF(iso)4E*-2 in the edited lines was observed in mock- and WSMV- inoculated plants (Figure 4.7 A and B). The relative expression of *eIF4G* and the isoforms *eIF(iso)4G-1* and *4G-2* was similar in mock- and WSMV- inoculated plants, but the relative expression of the genes *eIF4E-1* and *nCBP* increased in WSMV- inoculated plants (Figure 4.7 A and B). Despite the reduction in expression levels of the target gene *eIF(iso)4E*-2, plants of the CRISPR/Cas9-edited lines developed characteristic symptoms of the disease as did the Bobwhite_WT plants (Figure 4.8 A). Significant differences in the virus titer, measured as the relative expression of the WSMV *NIa* gene in infected plants, among the CRISPR/Cas9-edited, Bobwhite_WT and 'RonL' were not detected (Figure 4.9 A). Although 'RonL' is resistant to WSMV (*Wsm2* gene), plants from this material developed symptoms, which suggested a potential problem with the temperature of the growth chamber. After the bioassay it was found that although the temperature was set at 18 ºC, the actual temperature was close to 20ºC, and *Wsm2* is no longer effective at this temperature.

In the ***Bioassay 2***, the response to WSMV isolate 'Sidney 81' was evaluated in T_2 plants from the *eIF4G* edited lines 5697.A.5 (wt/wt, B: wt/wt, D: +1/+1), 5697.A.12 (A: +1/+1, B: wt/wt, D: +1/+1), 5697.B.9/5697.B.12 (A: +1/+1, B: wt/wt, D: wt/wt), and controls Bobwhite_WT and 'RonL'. The relative expression of the target gene *eIF4G* and other isoforms was evaluated in WSMV-inoculated plants from the CRISPR/Cas9 edited lines and Bobwhite_WT. As presented in the Figure 4.7 C, a slight reduction in the expression of the targeted gene *eIF4G* was observed in the edited lines when compared to Bobwhite_WT, especially in the line 5697.A.12, nevertheless the differences in the relative expression were not significant. No significant differences were observed in the levels of expression of other translation initiation factor isoforms. Plants from the three CRISPR/Cas9-edited lines developed characteristic yellow streaks in leaves (Figure 4.8 B), but a reduction in the virus titer was detected in plants from the lines 5697.A.12 and 5697.B12 (Figure 4.9 B). Plants from the material 'RonL' showed a reduced virus titer, ratifying that a the proper temperature (18 ºC or below) the gene *Wsm2* confers resistance to WSMV.

In the ***Bioassay 3***, T_3 plants from the *eIF(iso)4E-2*-edited lines 4385.A.5.3, 4E2.30_4385.B.12.1, 4E2.30_4385.B.16.1, T_2 plants from the e*IF4G*-edited lines 5697.A.5, 4G_5697.A.12, and 4G_5697.B.12, *eIF(iso)4E-2*-RNAi silenced line 1550.A.3.2.1 (T_4) and *eIF4G*-RNAi silenced line 1673.A.3.2 (T_3) were challenged with two WSMV isolates, 'Sidney 81', used in the previous bioassays, and 'MHK', used by Rupp et al. (2019). Two weeks after the second inoculation, visual evaluation of these plants showed that both WSMV isolates, 'Sidney 81' and 'MHK', infected plants from the CRISPR/Cas9 edited lines and plants from the 1673.A.3.2 line (Figure 4.10). Plants with the *eIF4G* gene silenced or edited showed more severe symptoms than the other plants, including Bobwhite_WT, whereas plants from the RNAi-

silenced line 1550.A.3.2.1 were slightly less affected by both WSMV isolates (Figure 4.10). Samples from this bioassay were not analyzed by RT-qPCR, but samples were collected and stored at -80ºC.

Discussion

CRISPR-mediated genome editing has been used to generate resistance to viruses in several pathosystems, by introducing targeted mutations in host plant genes, or by targeting viral genomes directly (Kalinina, et al. 2020). Host genome editing has focused primarily on the inactivation of the transcription factors eIF4E/eIF4G or their isoforms (Chandrasekaran et al., 2016; Gomez et al., 2019; Pyott et al. 2016), or the creation of new variants of these factors (Bastet et al., 2019; Macovei et al., 2018). In this study, embryogenic calli of the susceptible wheat cv. 'Bobwhite' were co-bombarded with DNA plasmids carrying *Cas9*, *sg_RNA*, and *bar* genes. sg_RNAs were designed to target four different sites in each of the *eIF(iso)4E-2* and *eIF4G* genes, with the final goal of knockout these genes and generate resistance to WSMV. From 102 positive transgenic plants carrying *Cas9* and *sgRNA*, only one T_0 plant with mutations in *eIF(iso)4E-2*, and 3 T_0 plants with mutations in different target sites of *eIF4G* were recovered. Editing efficiencies for the four sgRNAs were between 6.66% and 11.11%. These efficiencies were consistent with what was previously reported for CRISPR-editing in wheat, for example, previous wheat-genome editing experiments conducted in the Plant Transformation Laboratory at Kansas State University reported editing efficiencies ranging from 6.6% to 10% (Su et al., 2019; Wang et al., 2018; Wang et al., 2019), and a review of the application of CRISPR-based genome editing in wheat reported editing efficiencies between 1% to 9.5%, when particle-bombardment was used to deliver the CRISPR/Cas components (Kumar et al., 2019).

Mutations in the six alleles of *eIF(iso)4E-2* were detected in the T_0 plant 4385, and although mutations were bi-allelic (each allele in each genome had a different mutation), plants with homozygous mutations in each genome were recovered in the T_1 generation. Relative expression analyses of *eIF(iso)4E-2* and other factors in T_2 plants from three edited lines (4385.A.5, 4385.B.12 and 4385.B.16) showed a strong reduction in the expression of the targeted gene, but not significant differences in the expression of the other factors, when compared to Bobwhite_wild-type plants. The decrease in amount of *eIF(iso)4E-2* mRNAs in the edited lines could be result of the degradation of the mutated mRNAs with premature termination codons (PTC) by the Nonsense-mediated mRNA decay (NMD) pathway. NMD is one of the surveillance mechanisms that eukaryotic cells have to prevent the synthesis of aberrant proteins by the degradation of mRNAs that have acquired PTC due to mutations or mRNAs with errors in splicing (Brogna & Wen, 2009; Hung et al., 2016). The level of expression of the three binding proteins, *eIF4E*, *eIF(iso)4E-2* and *nCBP*, in the control Bobwhite_WT, showed that *eIF(iso)4E-2* transcripts were less abundant, which could explain why the knockout of this isoform does not have negative effects on the phenotype of the *eIF(iso)4E-2* edited lines, although it has not been established what is the abundance of the factors eIF4F and eIF(iso)4F in wheat at different development stages or in different tissues. Alternatively, it has been suggested that there is some redundancy in the function of *eIF4E* and *eIF(iso)4E*, since no altered phenotypes were observed in natural or induced knockout mutants (Bastet et al., 2017; Dinkova et al., 2016), and the knockout of *eIF(iso)4E* in *Arabidopsis* resulted in an increased expression of *eIF4E* (Duprat et al., 2002), maybe as a mechanism to compensate the translation in the plant. In the *eIF(iso)4E-2* edited lines, no increase in the expression of the other forms was observed, so redundancy in the

function or a generalized low abundance of the isoform would be valid explanations of why no negative effects were observed in the knockout mutants.

Mutations in the six alleles of the *eIF4G* gene were not recovered. The 5697 T_0 plant showed heterozygous mutations in the A and D genomes, and only two T_1 plants with homozygous mutations in both genomes were recovered, whereas alleles of the B genome were wild-type. It is possible that knockout of the six eIF4G alleles has deleterious effects on wheat development. A similar situation was reported in rice, where mutated alleles resulting in truncated eIF4G were not found in homozygous state (Macovei et al., 2018). The relative expression of the genes encoding the different factors eIF4F/eIF(iso)4F were evaluated in 5697 lines inoculated with WSMV. A slight reduction in the relative expression of *eIF4G* in T_2 plants of the mutant lines 5697.A.5, 5697.A.12, and 5697.B.9 was observed, when compared to Bobwhite_WT, and as expected, the expression in line 5697.A.12 was lower than in the other lines, since in this line four alleles are mutated. The relative expression of the genes encoding the different factors should be tested in non-inoculated or mock-inoculated T_2 plants from the lines 5697 to confirm the observed results. Altered phenotype has not been seen in non-inoculated plants from the line 5697.A.12, which suggest that eIF4G alleles in the B genome, or the isoforms eIF(iso)4G-1 and eIF(iso)4G-2 are enough to maintain the translation in these plants. Plants from the lines 4385 and 5697 have been kept under growth chambers conditions, therefore it is unknown if the edition of *eIF(iso)4E-2* and *eIF4G* may compromise the development or response of these plants to external factors when they are under uncontrolled conditions.

Resistance to the isolate ‘Sidney 81’ of WSMV was not observed in any of the CRISPR/Cas9 edited lines, although a slight reduction in virus titer was observed in T_2 plants from the lines 5697.A.12 and 5697.B.12. This is the opposite of the results reported by Rupp et

al. (2019), where the silencing of *eIF(iso)4E-2* and *eIF4G* genes by RNAi resulted in a decrease in the accumulation of WSMV, as well as TriMV, and SbWMV. Rupp et al. (2019) used a different WSMV isolate in their experiments, the 'MHK' isolate, but preliminary results obtained in the present study with the isolate 'MHK' suggested that it also successfully infected the CRISPR/Cas9- edited lines. Some viruses or virus strains preferentially use eIF4E, eIF(iso)4E, eIF4G or eIF(iso)4G factors to complete their infection cycle, whereas some others seem to have no preference (Dinkova et al., 2016; Schmitt-Keichinger, 2019), moreover, it has been found that a new player, the isoform nCBP, whose function in the eukaryotic cell is not yet fully understood, has an important role in resistance to viruses in cassava and *Arabidopsis* (Gomez et al., 2019; Keima et al., 2017). To date there are no known *in vitro* studies that have reported which components of the translation initiation complexes are involved in the translation of the WSMV genome. Results presented by Rupp et al. (2019) suggested that WSMV uses components of both eIF4F complexes, since the silencing of a factor in each complex (*eIF4G* from the eIF4F complex, and *eIF(iso)4E-2* from the eIF(iso)4F complex) resulted in less accumulation of the virus. Results from the present study also suggested that WSMV could be interacting with more factors than *eIF(iso)4E-2* or *eIF4G.* Protein-protein interaction assays, like yeast-two hybrid (Y2H), co-immunoprecipitation (Co-IP), or bimolecular fluorescent complementation (BiFC) could give some clues about what factors interact with the VPg of WSMV, although, eIF4F factors are not only involved with the translation of the viral RNA, but also in cell-to-cell movement (Gao et al., 2004; Keima et al., 2017) and systemic spread (Contreras-Paredes et al., 2013). Contrary to the limited knowledge about WSMV translation, an *in vitro* study suggested that TriMV interacts with high affinity with eIF4G/eIF (iso)4G than with eIF4E/eIF(iso)4E, and that the translation is independent of eIF4E but requires eIF4G (Roberts et

al., 2017). It has also been reported that the translation of BYDV is mediated by the interaction between the cap-independent translation enhancer (CITE) of BYDW (called BTE) and eIF4G (Treder et al., 2008). The response of CRISPR/cas9 edited line 5697.A.12 to TriMV and BYDV should be evaluated to confirm the role of eIF4G in the translation of these two viruses.

Some authors have suggested that knockout mutations could induce virus to hijack other isoforms (Bastet et al., 2017; Gauffier et al., 2016), or that viruses can overcome knockout resistance by using eIF4F-independent pathways (Gallois et al., 2010). This could explain why gene knockdown (mediated by RNAi), but not knockout, had an effect on WSMV accumulation. Analysis of the progeny of genetic crosses between CRISPR/Cas9 edited plants and RNAi silenced plants could help determine whether loss-of-function alleles induces WSMV to use other factors. WSMV can either adapt to using other factors when the "preferred" factor is not available or it uses components of both eIF4F complexes. If either is true, the resistance to this virus cannot be addressed by the knockout of a specific factor (as reported in this study), nor by accumulation of loss-of-function factors, as this would have negative effects on the plant. In addition, it has been reported that resistance conferred by knockout of an eIF4F factor is generally narrower than natural resistance and it can be broken by new virus variants (Gauffier et al., 2016; Gallois et al., 2010). This is particularly important, as new WSMV variants have been identified in wheat fields, overcoming the resistance conferred by the *Wsm2* resistance gene (Fellers et al., 2019), suggesting that virus populations could adapt to resistance generated by the loss-of-function of a susceptibility gene. An alternative would be to design variants of the factors with which WSMV interacts, by generating point-mutations but without inducing loss-of-function of these factors. Macovei et al. (2018) showed that in-frame mutations induced by CRISPR/Cas9 in *eIF4G* conferred resistance to *Rice tungro spherical virus* (RTSV) and *Rice*

tungro bacilliform virus in rice, whereas plants with other types of mutations were susceptible or not viable. Bastet et al. (2019) used CRISPR-Cas9 cytidine deaminase to introduce a mutation in the wild-type *eIF4E* gene of *Arabidopsis*, conferring resistance to *Clover yellow vein virus* (ClYVV), then, the CRISPR/Cas base editing technology could be a valuable tool to incorporate broad virus resistance into wheat.

This study showed that the CRISPR/Cas9 system was useful for inducing mutations in the *eIF(iso)4E-2* and *eIF4G* genes in hexaploid wheat. The results suggested that eIF4G is essential for normal wheat development, but not the eIF(iso)4E-2 isoform. With the results obtained in this study it was not possible to conclude whether these two factors play a role or not in WSMV infection. There are still many questions to be resolved about the dynamics of wheat translation initiation factors and their interaction with WSMV. For example, it is not clear if in wheat plants there are differences in the abundance of the components of the eIF4F and eIF(iso)4F complexes, nor if the alleles of the different genomes have a different contribution to this abundance, but this could be assessed by using third or fourth generation sequencing technologies. It is not known if the hijacking of the factors by WSMV can limit their accessibility for the translation of host mRNAs and therefore induce changes in the levels of expression of the different factors. In this study it was observed that the expression of the *nCBP* isoform increased in WSMV inoculated plants, but this response must be validated. Finally, the identification of which factors interact with WSMV is fundamental, and as mentioned earlier, this can be evaluated through different protein-protein assays. The development of strategies aimed at creating resistance by CRISPR-mediated genome editing has great potential, but it requires a better understanding of the WSMV – wheat interaction.

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Figure 4.1 Comparisons of the chromatograms of the regions flanking the target sites in Bobwhite_wild type plants and in the putative mutant plants.

Possible indels at the target site in some of the transgenic plant alleles result in a mixture of wild-type and mutated sequences that are visualized as double peaks in the chromatograms

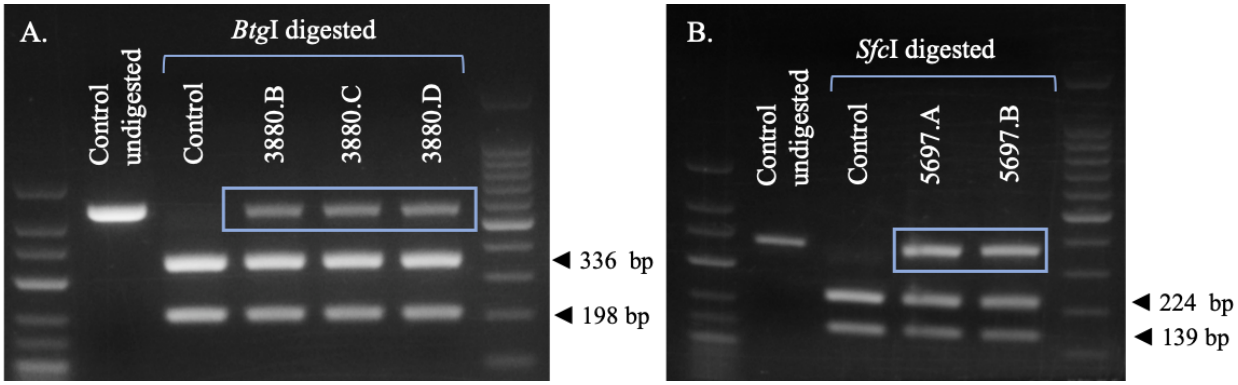


Figure 4.2 Electrophoresis analysis of *Btg*I- and *Sfc*I- digested PCR products from putative mutant plants.

Digested control (Bobwhite_wild-type) showed the two fragments resulting from the complete digestion of the amplicon, whereas the tillers from the mutant plant 3880 (A) and 5597 (B) showed the two fragments resulting from the digestion plus an additional fragment corresponding to the undigested amplicon (blue square).

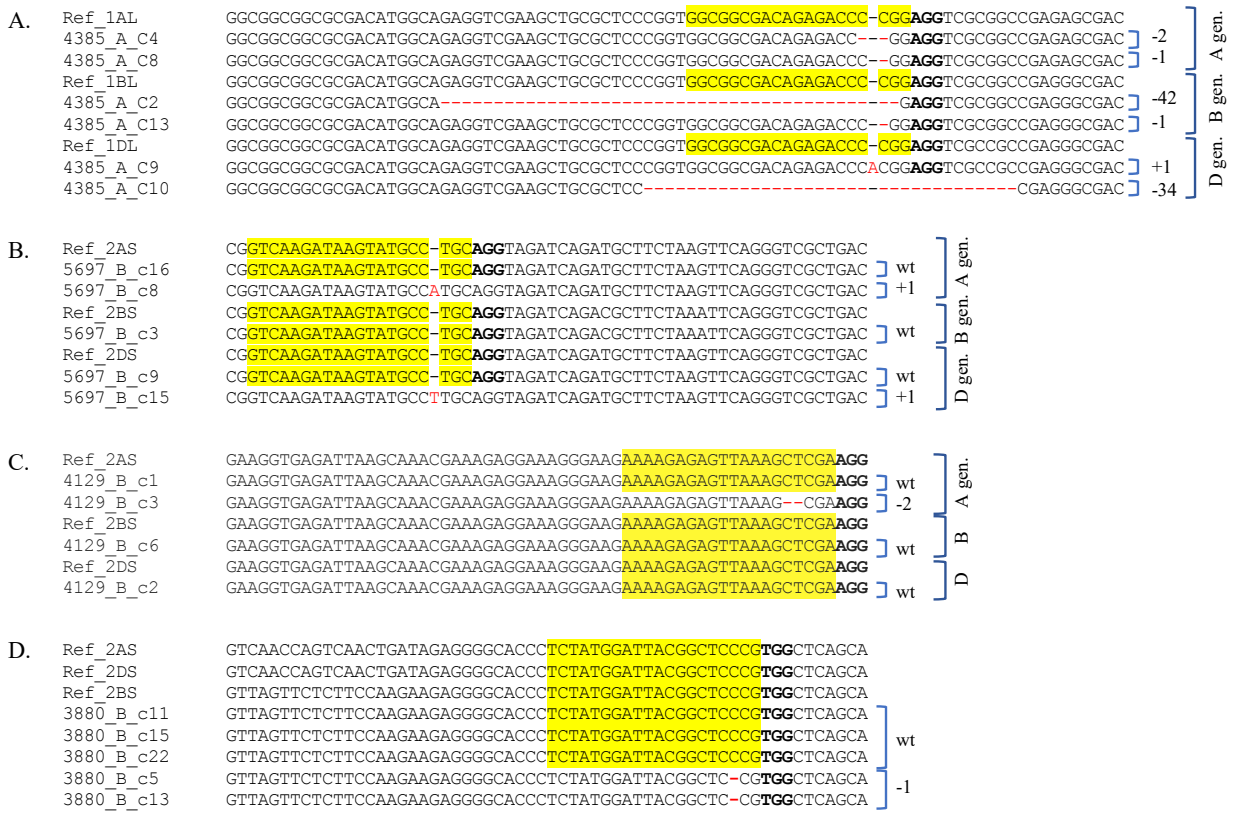


Figure 4.3 Mutations identified in the target sites of the four T_0 mutant plants.

(A) target site 4E2.30 in the T_0 plant 4385, (B) target site 4G.523 in the T_0 plant 5697, (C) target site 4G.2999 in the T_0 plant 4129, and (D) target site 4G.3454 in the T_0 plant 3880. The wild-type sequence of the target site is highlighted in yellow, and the PAM sequence is in bold. Only a fragment of the complete sequence is shown here.

A.

```
Ref_Q03389_IF4E2      MAEVEAALPVAATETPEVAAEGDAGAAEAKGPHKLQRQWTFWYDIQTKPKPGAAWGTSLK  60
4385_1AL(-1 del)      MAEVEAALPVAATETRRSRPRATPVRPRRRGRTSCSGSGPSGTTSRPSPSPAPPGAPRSK  60
4385_1AL(-2 del)      MAEVEAALPVAATETGGRGRERRRCGRGEGAAQAAAAVDLLVRHPDQAQARRRLGHLAQK  60
4385_1BL(-42 del)     MAEVAA--------------EGDAGAAEAKGPHKLQRQWTFWYDIQTKPKPGAAWGTSLK  46
4385_1BL(-1 del)      MAEVEAALPVAATETRRSRPRATRVRPRRRGRTSCSGSGPSGTTSRPSPSPAPPGAPRSK  60
4385_1DL(-34 del)     MAEVEAALPRATRVRPRRRGRTSCSGSGPSGTTSRPSPSPAPPGAPRSKRATPSTPSKSS  60
4384_1DL(+1 ins)      MAEVEAALPVAATETHGGRRRGRRGCGRGEGAAQAAAAVDLLVRHPDQAQARRRLGHLAQ  60

Ref_Q03389_IF4E2      KGYTFDTVEEFWCLYDQIFRPSKLVGSADFHLFKAGVEPKWEDPECANGGKWTVISSRKT  120
4385_1AL(-1 del)      RATPSTPSKSSGACMIRFSVRVSW/----------------------------------  84
4385_1AL(-2 del)      GLHLRHRRRVLVLV/--------------------------------------------  74
4385_1BL(-42 del)     KGYTFDTVEEFWCLYDQIFRPSKLVGSADFHLFKAGVEPKWEDPECANGGKWTVISSRKA  106
4385_1BL(-1 del)      RATPSTPSKSSGACMIRFSVRVSW/----------------------------------  84
4385_1DL(-34 del)     GACMIRFSVRVSW/---------------------------------------------  73
4384_1DL(+1 ins)      KGLHLRHRRRVLVLVSDFPSE/-------------------------------------  81

Ref_Q03389_IF4E2      NLDTMWLETCMALIGEQFDESQEICGVVASVRQRQDKLSLWTKTASNEAVQVDIGKKWKE  180
4385_1AL(-1 del)      ------------------------------------------------------------
4385_1AL(-2 del)      ------------------------------------------------------------
4385_1BL(-42 del)     NLDTMWLETCMALIGEQFDESQEICGVVASVRQRQDKLSLWTKTASNEAVQVDIGKKWKE  166
4385_1BL(-1 del)      ------------------------------------------------------------
4385_1DL(-34 del)     ------------------------------------------------------------
4384_1DL(+1 ins)      ------------------------------------------------------------

Ref_Q03389_IF4E2      VIDYNDKMVYSFHDDSRSQKPSRGGRYTV  209
4385_1AL(-1 del)      ----------------------------
4385_1AL(-2 del)      ----------------------------
4385_1BL(-42 del)     VIDYNDKMVYSFHDDSRSQKPSRGGRYTV  195
4385_1BL(-1 del)      ----------------------------
4385_1DL(-34 del)     ----------------------------
4384_1DL(+1 ins)      ----------------------------
```

B.

```
Ref_G5CEW6_IF4G       ATQAGQSIPFMNPSMSNTVPASHKDNIAGPAPSGQSQLIGKPQGGLHMEKPVPSVKISMP  180
5697_2AS(+1 ins)      ATQAGQSIPFMNPSMSNTVPASHKDNIAGPATSGQSQLIGKPQGGLHMEKPVPSVKISMP  180
5697_2DS(+1 ins)      ATQAGQSIPFMNPSMSNTVPASHKDNIAGPATSGQSQLIGKPQGGLHMEKPVPSVKISMP  180
                      ****************************** ****************************

Ref_G5CEW6_IF4G       AGRSDASKFRVADHAVQHRQKDNEVISGAMVSNKPVSEKESKAPSIPEKHSKESKAPSAV  240
5697_2AS(+1 ins)      CR/-------------------------------------------------------  182
5697_2DS(+1 ins)      CR/-------------------------------------------------------  182
```

C.

```
Ref_G5CEW6_IF4G       FDKLFEQVKEVNIDNVSTLTGVISQIFDKALMEPTFCEMYANFCSHLAGALPDFSEDNEK  957
4129_2AS(-2 del)      FDKLFEQVKEVNIDNVSTLTGVISQIFDKALMEPTFCEMYANFCSHLAGALPDFSEDNEK  960
                      ************************************************************

Ref_G5CEW6_IF4G       ITFKRLLLNKCQEEFERGEREEAEADKTEEEGEIKQTKEEREEKRVKARRRMLGNIRLIG  1017
4129_2AS(-2 del)      ITFKRLLLNKCQEEFERGEREEAEADKTEEEGEIKQTKEEREEKRVKAKAHAG/------  1013
                      ************************************************: :
```

D.

```
Ref_G5CEW6_IF4G       RSRGPVVSSLPRRGAPSMDYGSRGSAAPLVSPGPQQRGRGFGNQDIRYEQERHQFDRTVP  1195
3880_2AS(-1 del)      RSRGPVVSSLPSRGAPSMDYGSVAQQHHWYLQVLSNEGVDLVIKIFGMSRKGISLIELFP  1200
                      *********** ********** ..         .:.* .:  : :  .::  .: . .*

Ref_G5CEW6_IF4G       LPQRSVKDEAITLGPQGGLARGMSLRGQPPVSNSELPSVVDQRRILSGPNGYNSVPSTTR  1255
3880_2AS(-1 del)      FPSVL/----------------------------------------------------  1205
                      :*.
```

Figure 4.4 Alignment of the wild-type proteins eIF(iso)4E-2 and eIF4G with the predicted mutated proteins produced by the plants 4385 (A), 5697 (B), 4129 (C) and 3880 (D).

Indels in the target sites would produce changes in the reading frame, generating short proteins due to the creation of early termination codons (represented by $\neq$) and proteins with amino-acid substitutions (in blue font). The protein resulting from the *eIF(iso)4E-2* gene with the -42 deletion would have a deletion of 14 a.a. at the N terminal of the protein. The wild-type proteins eIF(iso)4E-2 and eIF4G are 209 a.a and 1488 a.a. length, respectively. Wild-type amino-acid sequences are in black font.

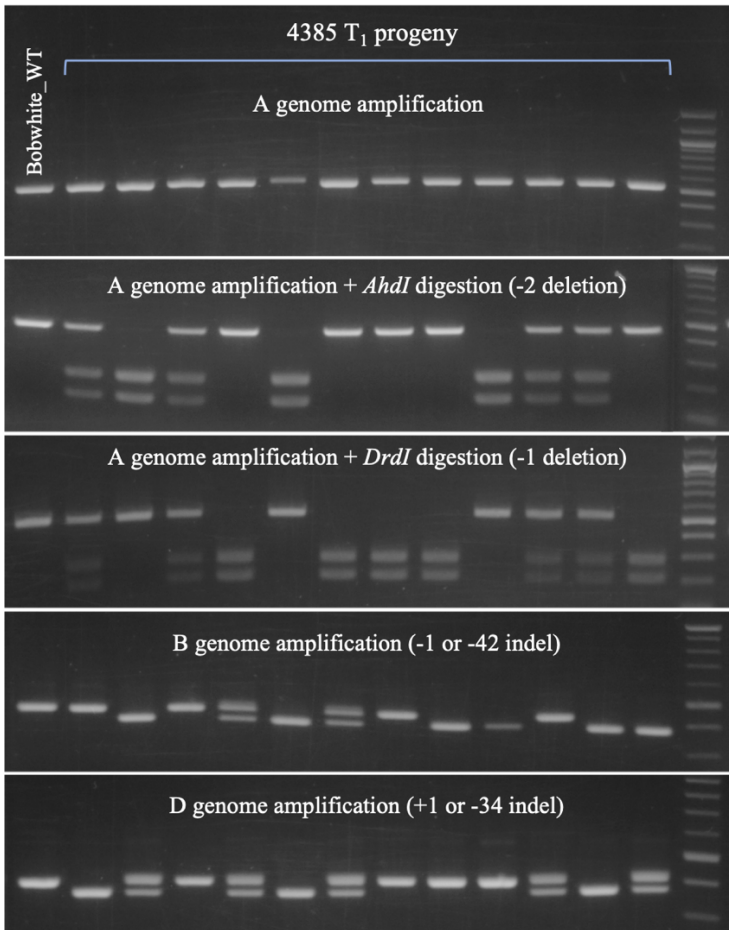


Figure 4.5 Characterization of mutations in the target site 4E2.30 in the T_1 progeny of the plant 4385.

The flanking region in the genome A was amplified with specific primers and the PCR products were digested with the restriction enzymes *Ahd*I and *Drd*I to identify -2 and -1 deletions, respectively. Complete digestion with *Ahd*I represented homozygous mutation -2/-2, complete digestion with *Drd*I represented homozygous mutation -1/-1, incomplete digestion with both enzymes represented heterozygous mutations (-1/-2). Flanking regions in the B and D genomes were amplified with specific primers, differences in amplicon size allowed the identification of small indels (-1 or +1) or large deletions (-42 or -34). The -1/-1 deletion in the B genome was

confirmed by digestion of the amplicon with the enzyme *Drd*I (not shown here), and the +1/+1 deletion in the D genome was confirmed by sequencing.

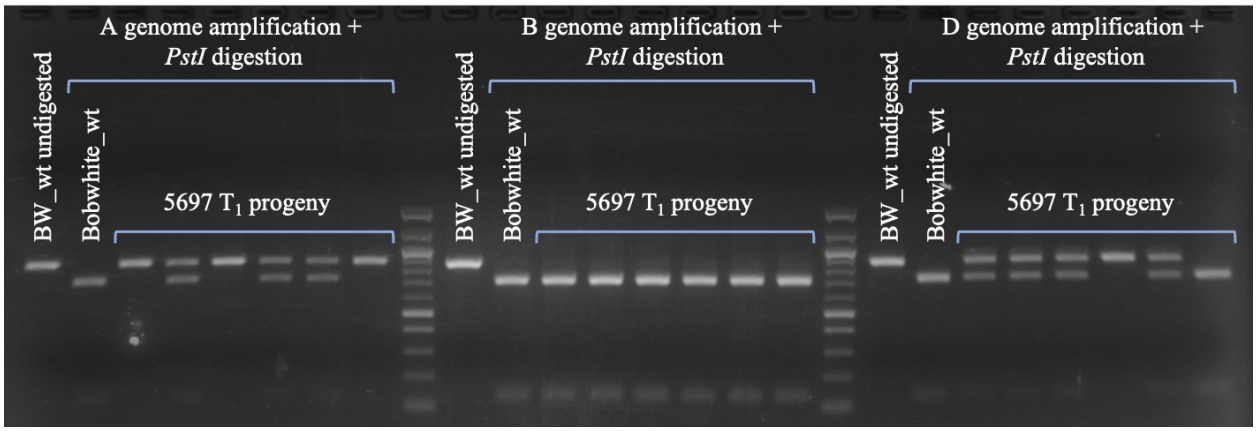
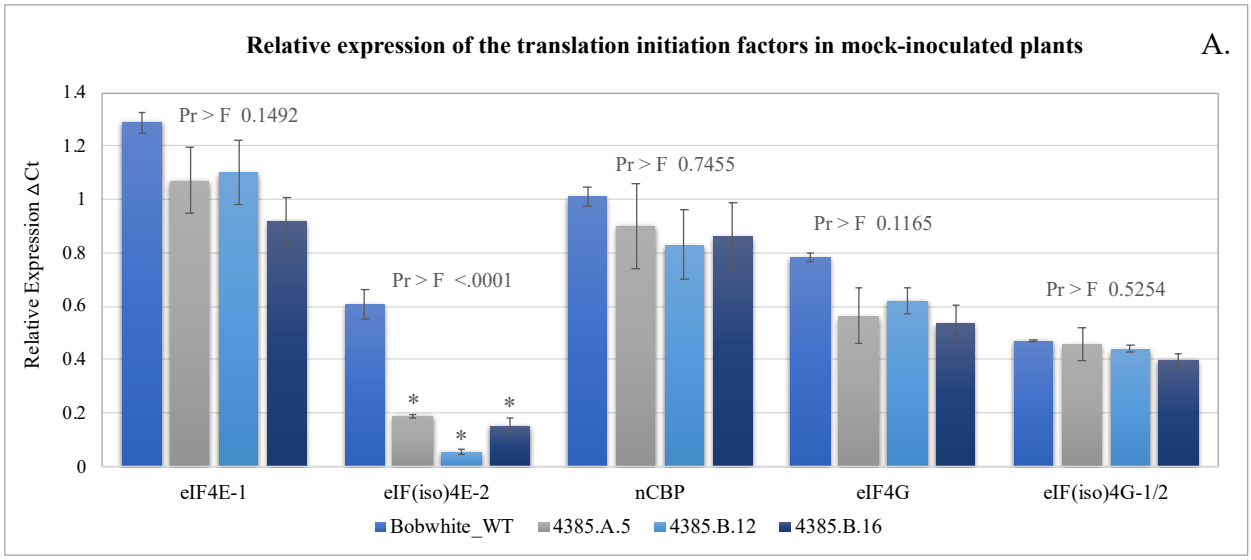
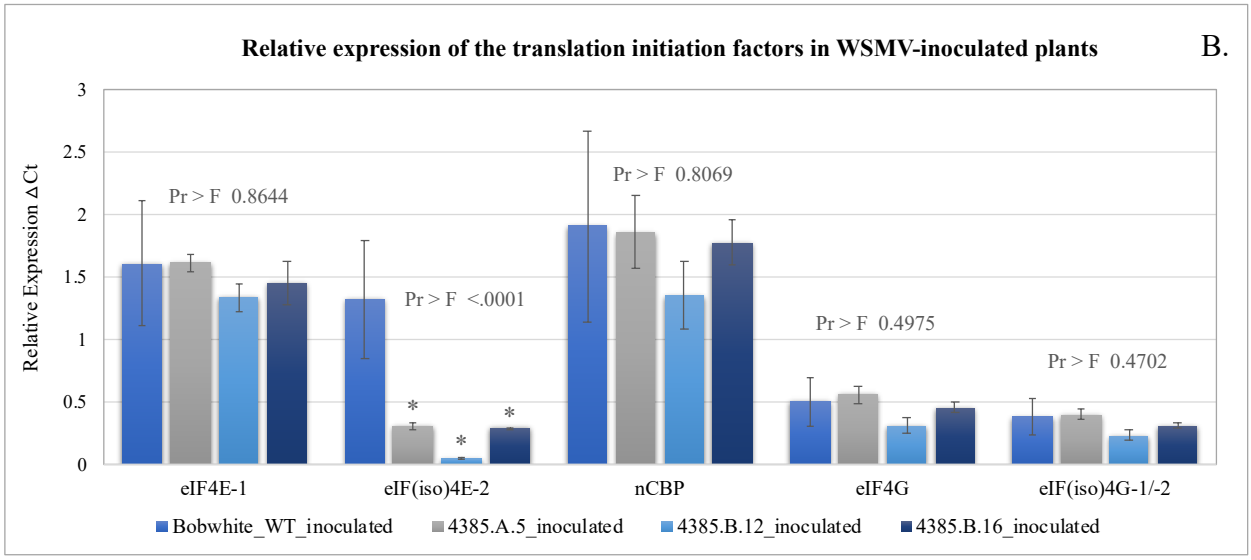


Figure 4.6 Characterization of mutations in the target site 4G.523 in the T_1 progeny of the plant 5697.

Regions flanking the target site in the A, B and D genome were amplified using genome-specific primers, and amplicons were digested with the restriction enzyme *Pst*I. The enzyme recognition site is lost due to sequence modifications, the complete digestion of the product indicated that both alleles in the specific genome are wild-type, three bands represented heterozygous mutations (+1/wt), and undigested products represented homozygous mutations (+1/+1).


Relative expression of the translation initiation factors in mock-inoculated plants
A.
Relative Expression ΔCt
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Pr > F 0.1492
Pr > F <.0001
Pr > F 0.7455
Pr > F 0.1165
Pr > F 0.5254
*
*
*
eIF4E-1
eIF(iso)4E-2
nCBP
eIF4G
eIF(iso)4G-1/2
Bobwhite_WT
4385.A.5
4385.B.12
4385.B.16


Relative expression of the translation initiation factors in WSMV-inoculated plants
B.
Relative Expression ΔCt
3
2.5
2
1.5
1
0.5
0
Pr > F 0.8644
Pr > F <.0001
Pr > F 0.8069
Pr > F 0.4975
Pr > F 0.4702
*
*
*
eIF4E-1
eIF(iso)4E-2
nCBP
eIF4G
eIF(iso)4G-1/-2
Bobwhite_WT_inoculated
4385.A.5_inoculated
4385.B.12_inoculated
4385.B.16_inoculated

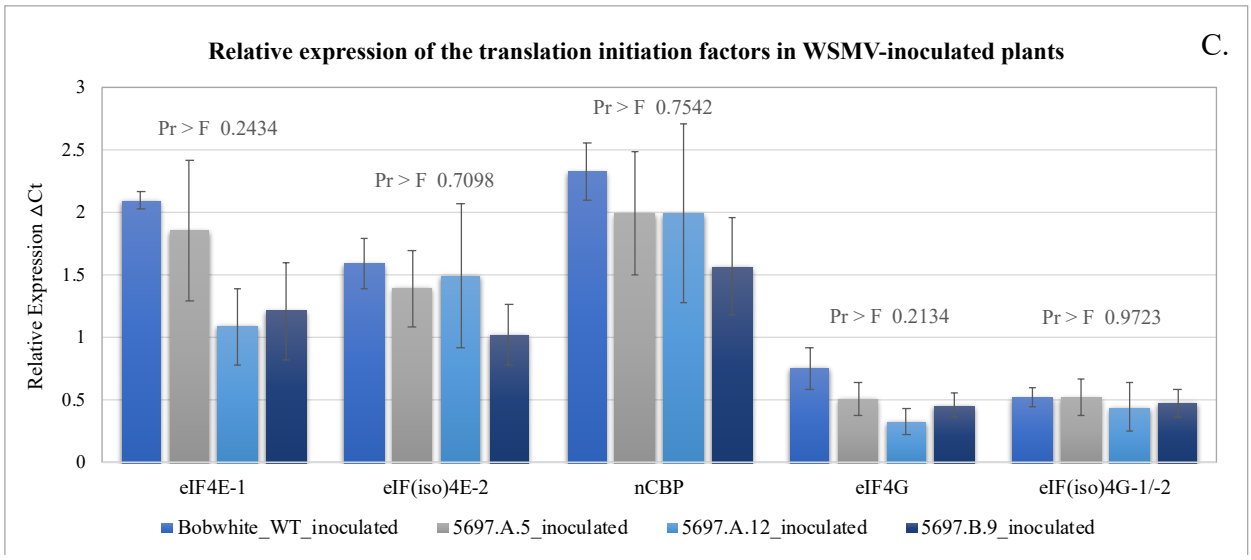

Relative expression of the translation initiation factors in WSMV-inoculated plants
C.
Relative Expression ΔCt
3
2.5
2
1.5
1
0.5
0
Pr > F 0.2434
Pr > F 0.7098
Pr > F 0.7542
Pr > F 0.2134
Pr > F 0.9723
eIF4E-1
eIF(iso)4E-2
nCBP
eIF4G
eIF(iso)4G-1/-2
Bobwhite_WT_inoculated
5697.A.5_inoculated
5697.A.12_inoculated
5697.B.9_inoculated

Figure 4.7 Relative expression of the *eIF4E* and *eIF4G* isoforms in CRISPR/Cas9 edited plants. (A) mock-inoculated *eIF(iso)4E-2*-CRISPR/Cas9-edited lines and Bobwhite_WT, (B) WSMV-inoculated *eIF(iso)4E-2*-CRISPR/Cas9-edited lines and Bobwhite_WT, and (C) WSMV-inoculated *eIF4G*-CRISPR/Cas9-edited lines and and Bobwhite_WT. The relative expression of the translation initiation factors was calculated with the ΔCt method, using *actin* as reference gene. Three biological replicates with three technical repetitions were used. The expression levels were compared by one-way analysis of variance (ANOVA), and means were compared to the control Bobwhite_WT using Dunnet's test (* if significant differences with the control).

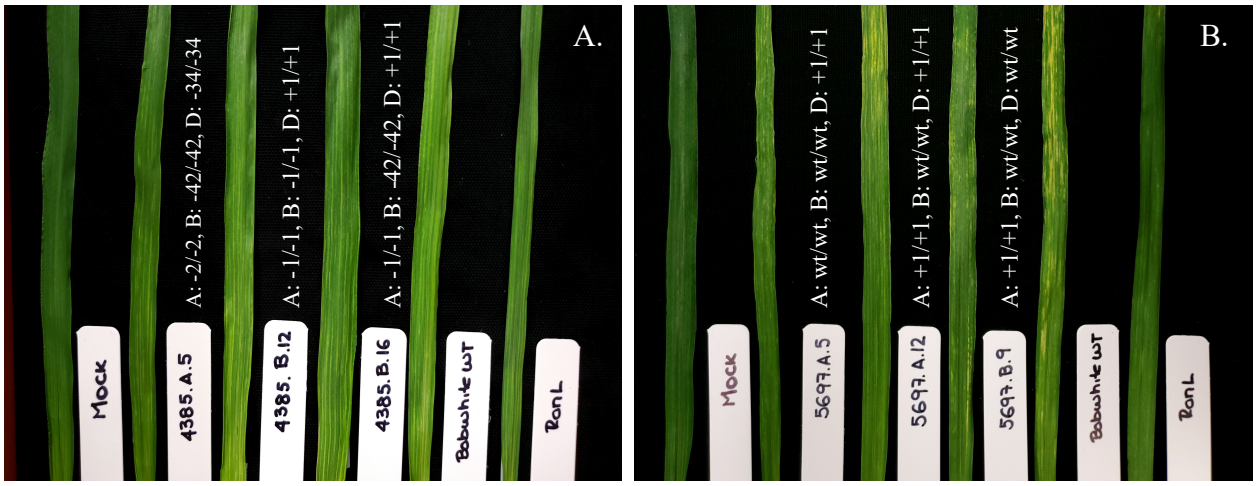


Figure 4.8 Characteristic yellow streaks and mosaics caused by WSMV isolate 'Sidney 81' in leaves of the (A) *eIF(iso)4E-2*-CRISPR/Cas9 edited lines, and (B) *eIF4G*-CRISPR/Cas9 edited lines.

Controls Bobwhite mock-inoculated, Bobwhite_WT inoculated and RonL_inoculated were included as reference. Genotypes of the CRISPR/Cas9 edited lines were also included. Photos were taken 17 days after the second inoculation (A), and 14 days after the second inoculation (B).

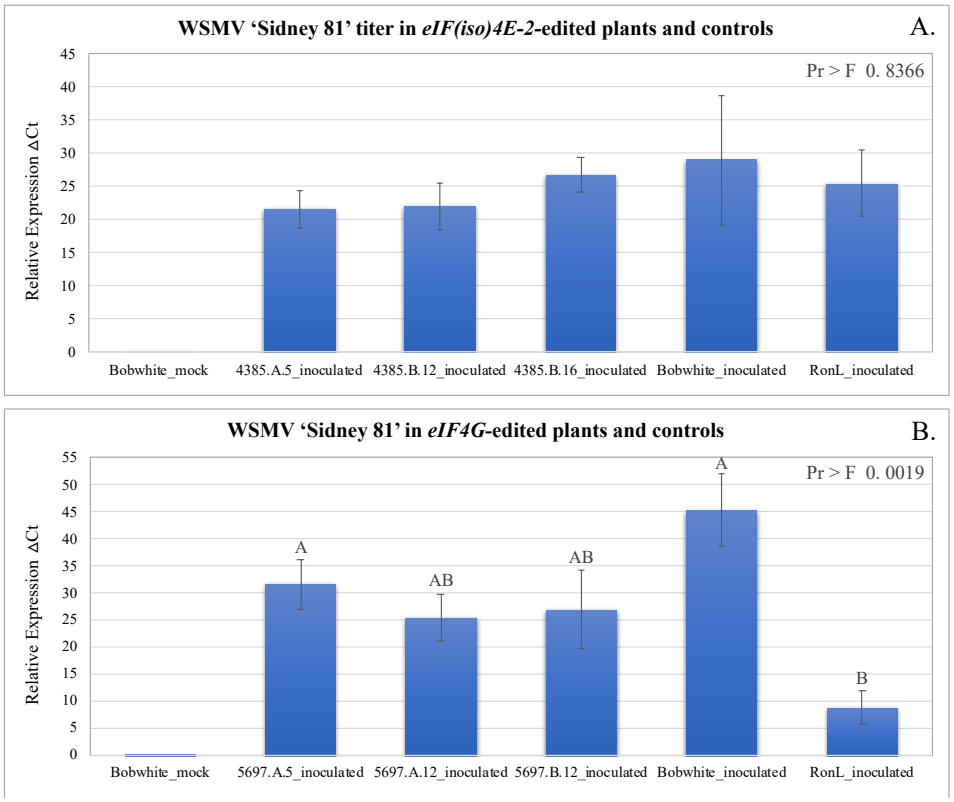


Figure 4.9 WSMV concentration (titer) in (A) inoculated *eIF(iso)4E-2*-CRISPR/Cas9-edited lines and control plants, and in (B) inoculated *eIF4G*-CRISPR/Cas9-edited lines and controls. The relative expression of the *NIa* gene was calculated with the ΔCt method, using *actin* as reference gene. Six biological replicates with three technical repetitions were used. The expression levels were compared by one-way analysis of variance (ANOVA), and multiple comparisons were done using the Ryan-Einot-Gabriel-Welsch F (REGWF) test.

Figure 4.10 Characteristic yellow streaks caused by the WSMV isolates 'Sidney 81' and 'MHK' in CRISPR/Cas9 edited lines, RNAi-silenced lines and controls.

Picture was taken 14 days after the second inoculation.

Table 4.1 Characteristics of the sites identified in the *eIF(iso)4E-2* and *eIF4G* genes to be targeted by the CRISPR/Cas9 system, including the PAM (in bold).

[1]RE_sites: restriction enzyme recognition sites inside the target sequence; [2]hit_20mer: number of sites in the genome with homology to the 20 base-pair target sequence; [3]hit_12mer: number of sites in the genome with homology to the PAM-proximal 12 nucleotides located in the 3' end of the target sequence; [4]Region used for RNAi: the target sequence is located inside the region used to construct the RNAi harpin.

[CRISPRdirect | 2017-03-28 08:14:28]
sequence_name: **AAA34296.1_eIF(iso)4E-2**
pam_sequence: NGG

# start	end	strand	sequence	GC	Tm	RE_sites[1]	hit_20mer[2]	hit_12mer[3]	Overlapping genes	Region used for RNAi[4]
30	52	+	GGCGGCGACAGAGACCCCGG**AGG**	80	85.99		3	6	No	No
163	185	+	TGGGGCACCTCGCTCAAAAA**GGG**	55	76.67	Bme1580I	3	11	No	No
332	354	+	AATGGACTGTGATATCTAGC**AGG**	40	67.44	EcoRV	3	10	Yes	Yes
456	478	-	**CCA**GAGACAGGATAAGCTTTCAT	40	67.07	HindIII	3	7	No	Yes

[CRISPRdirect | 2017-03-28 08:47:51]
sequence_name: **AEQ49596_eIF4G**
pam_sequence: NGG

# start	end	strand	sequence	GC	Tm	RE_sites[1]	hit_20mer[2]	hit_12mer[3]	Overlapping genes	Region used for RNAi[4]
523	545	+	GTCAAGATAAGTATGCCTGC**AGG**	45	69.36	PstI,SbfI,SfcI	3	7	No	No
2388	2410	-	**CCA**CATGGACTTGACAAACGGCC	55	73.59		3	9	No	No
2999	3021	+	AAAAGAGAGTTAAAGCTCGA**AGG**	35	64.65		3	8	No	No
3454	3476	+	TCTATGGATTACGGCTCCCG**TGG**	55	75.22	BtgI	3	9	No	Yes

Table 4.2 Sequences of the target-specific oligos.

Gene	# start	end	Oligo name	oligo sequence (5´- 3´) with overhangs
eIF(iso)4E-2	30	49	4E2_30_49F	CTTGGGCGGCGACAGAGACCCCGG
			4E2_30_49R	AAACCCGGGGTCTCTGTCGCCGCC
eIF(iso)4E-2	163	182	4E2_163_182F	CTTGTGGGGCACCTCGCTCAAAAA
			4E2_163_182R	AAACTTTTTGAGCGAGGTGCCCCA
eIF(iso)4E-2	332	351	4E2_332_351F	CTTGAATGGACTGTGATATCTAGC
			4E2_332_351R	AAACGCTAGATATCACAGTCCATT
eIF(iso)4E-2	459	478	4E2_459_478F	CTTGATGAAAGCTTATCCTGTCTC
			4E2_459_478R	AAACGAGACAGGATAAGCTTTCAT
eIF4G	523	542	4G_523_542F	CTTGGTCAAGATAAGTATGCCTGC
			4G_523_542R	AAACGCAGGCATACTTATCTTGAC
eIF4G	2391	2410	4G_2391_2410F	CTTGGGCCGTTTGTCAAGTCCATG
			4G_2391_2410R	AAACCATGGACTTGACAAACGGCC
eIF4G	2999	3018	4G_2999_3018F	CTTGAAAAGAGAGTTAAAGCTCGA
			4G_2999_3018R	AAACTCGAGCTTTAACTCTCTTTT
eIF4G	3454	3473	4G_3454_3473F	CTTGTCTATGGATTACGGCTCCCG
			4G_3454_3473R	AAACCGGGAGCCGTAATCCATAGA

Table 4.3 Primers used in PCR, semi-quantitative RT-PCR, and quantitative RT-qPCR assays.

Primer name	Sequence. (5'-3')	PCR product size (bp)	Description
M13_FWD M13_REV	GTAAAACGACGGCCAG CAGGAAACAGCTATGAC	166 + insert	Detection of TOPO vector with the insert
Tub-F Tub-R	ATCTGTGCCTTGACCGTATCAGG GACATCAACATTCAGAGCACCATC	500 gDNA / 409 cDNA	PCR and RT-PCR: control DNA contamination in cDNA samples
UbiABF BarABR	CCTGCCTTCATACGCTATTTATTTGC CTTCAGCAGGTGGGTGTAGAGCGTG	453	PCR - *bar* detection
TaCas9_FWD TaCas9_REV	GCGGCAGATAAGAAGTACAG ACCTTAGCCATCTCGTTAGA	281	PCR - *cas9* detection
TaU6_FWD	CCCAAGCTTGACCAAGCCCGTTATTCT	449 (with sgRNA_REV)	PCR - sgRNA detection
sgRNA_FWD sgRNA_REV	CTACGAGAGAGCTGAAGATAAC TCAAGTTGATAACGGACTAGC	327	PCR - sgRNA detection
30_F2	CTCATCCTCCCCATCCCAAA	322	Amplification of the region flanking the site 4E2.30
163_F 163_R3	ATGGCAGAGGTCGAAGCTG GATCAGATCTGAGCGGATCG	272	Amplification of the region flanking the site 4E2.163
332_F2 332_R3	CCGTCCGAGTAAGCTGGTAG AGCTTCGTTACTGGCAGTCT	399	Amplification of the region flanking the site 4E2.332
459_F2 459_R3	CATGTGGCTTGAAACGGTAA GATGCATGTTGATTGTGGAC	418	Amplification of the region flanking the site 4E2.459
523_F 523_R	CTATGTCAAATACTGTTCCTGCCA CTTSAATCGGTAAGGGTKGAGTC	363	Amplification of the region flanking the site 4G.523
2391_F 2391_R	AACCTCACCCAAGTTCTGCAAG TCATCAGAAACTTTGCCGACAA	370	Amplification of the region flanking the site 4G.2391
2999_F 2999_R	CTGCCAGACTTTAGTGAGGACA TGTTCCTTAGCCTTTGGATGATCT	371	Amplification of the region flanking the site 4G.2999
3454_F 3454_R	GAGGAGAACATTGAAGCACTAT CTGCCCTCTTAAAGACATACC	534	Amplification of the region flanking the site 4G.3454
1AL-30_F6	CTATCTCGCTTCACAACTAAAG	528 (with 163_R3)	Amplification of the region flanking the site 4E2.30 in the A genome
1BL_30_F3	TATGCCCGTGCGTGTAGCAAAG	422 (with 163_R3)	Amplification of the region flanking the site 4E2.30 in the B genome
1DL_30_F5	TCATAGCCTCATACCCACCT	414 (with 163_R3)	Amplification of the region flanking the site 4E2.30 in the D genome
2AS_523_R2	GACAATTCATCAATTCTTTCATTAACTTTTT	874 (with 523_F)	Amplification of the region flanking the site 4G.523 in the A genome
2BS_523_R3	CGCAATTCATTGATTCTTTCATTAACCTTTC	865 (with 523_F)	Amplification of the region flanking the site 4G.523 in the B genome
2DS_523_R4	CACAATTCATCCATTCTTTCATTAACTTCTA	874 (with 523_F)	Amplification of the region flanking the site 4G.523 in the D genome
2AS_3454_R2	CTACTCCCTCCGTCCGGAAATAC	1067 (with 3454_F)	Amplification of the region flanking the site 4G.3454 in the A genome
2AS_3454_R3	GATGACAAGTATTTTCGGACGGAG	971 (with 3454_F)	Amplification of the region flanking the site 4G.3454 in the A genome
2BS_3454_R3	TACATGTTCCAAAGAAATATAATAGAC	944 (with 3454_F)	Amplification of the region flanking the site 4G.3454 in the B genome
2DS_3454_R4	CTATGTTACAAAGAAAATATAGAC	947 (with 3454_F)	Amplification of the region flanking the site 4G.3454 in the D genome

Primer name	Sequence. (5'-3')	PCR product size (bp)	Description
qActin_2F	AGCTGGAGACTGCCAAGAAC	124	RT-qPCR - reference gene
qActin_2R	ATCATGGATGGCTGGAAGAG		
qIF4E1_2F	GCAGTGGAAGGAGTTTCTGG	101	RT-qPCR - relative expression of *eIF4E-1*
qIF4E1_2R	AAACGGTGTAGCGGTTCTTG		
qIF4E2_2F	CCAAAGTGGGAAGATCCAGA	111	RT-qPCR - relative expression of *eIF(iso)4E-2*
qIF4E2_2R	TCCAATCAGAGCCATACACG		
qIF4E3_2F	GAATCGGAATGCATCAGACC	108	RT-qPCR - relative expression of novel Cap-Binding Protein (nCBP)
qIF4E3_2R	GCGTCATGTGGYTTGTACTC		
qIF4G_1F	CCACATGGACTTGACAAACG	118	RT-qPCR - relative expression of *eIF4G* gene
qIF4G_1R	AGGAGCATTGGATTGTGGTC		
qIF4G1-2_1F	TGACCTGCGCAAGATTACTG	94	RT-qPCR - relative expression of *eIF4G-1* and *eIF4G-2*
qIF4G1-2_1R	AACCCAGCTCTGATCGTCAC		
qWSMV_2F	GGTGGAAAATCGTTGGGATG	99	RT-qPCR - WSMV titer
qWSMV_2R	GCAACTTCACGAACCTTGTC		

Table 4.4 Results obtained in bombardment experiments, number of positive transgenic plants and CRISPR/Cas9 edited plants.

Targeted gene	TaU6_sgRNA construct	No. calli bombarded	No. transgenic plants (with *cas9* and *sgRNA*)	No. plants with mutations (ID T0 plant)	Transformation efficiency	Editing efficiency
eIF(iso)4E-2	TaU6_eIF4E2.30_sgRNA	1175	9	1 (4385)	0.77	11.11
eIF(iso)4E-2	TaU6_eIF4E2.163_sgRNA	1000	18	0	1.80	
eIF(iso)4E-2	TaU6_eIF4E2.332_sgRNA	950	12	0	1.26	
eIF(iso)4E-2	TaU6_eIF4E2.459_sgRNA	1025	14	0	1.37	
eIF4G	TaU6_eIF4G.523_sgRNA	1200	12	1 (5697)	1.00	8.33
eIF4G	TaU6_eIF4G.2391_sgRNA	1050	7	0	0.67	
eIF4G	TaU6_eIF4G.2999_sgRNA	975	15	1 (4129)	1.54	6.66
eIF4G	TaU6_eIF4G.3454_sgRNA	1150	15	1 (3880)	1.30	6.66
	Total	8525	102	4	1.20	

Chapter 5 - Summary and Prospects

In this study, two biotechnological approaches were used with the aim to reduce wheat susceptibility to fungal and viral diseases. In the first approach, wheat was transformed with exogenous genes encoding antimicrobial peptides (AMPs) and a rice resistance (R) gene, with the purpose of enhancing resistance to Fusarium head blight (FHB) and wheat blast (WB). In the second strategy, CRISPR/Cas9 technology was used to knockout two wheat endogenous genes encoding the translation initiation factors eIF(iso)4E-2 and eIF4G, which act as susceptibility factors facilitating the translation of viral genomes.

Four genes encoding AMPs were selected, based on previous reports where their ability to reduce the growth of different plant pathogenic fungi was described. Wheat transgenic lines expressing independently the AMPs Ace-AMP1, WD, ARACIN1, and Zeamatin, were challenged with *Fusarium graminearum*, causal agent of fusarium head blight – FHB (Chapter 2) and *Magnaporthe oryzae Triticum* pathotype (MoT), causal agent of wheat blast (Chapter 3). From twenty transgenic lines tested with FHB, four lines showed a slight reduction in percentage of spikelets affected, but plants were considered susceptible because the percentages of affectation ranged between 68 and 86% at the end of the evaluation (14 or 16 days after the inoculation). Fourteen transgenic lines were tested against wheat spike blast (W_SB) and seven lines were assessed against wheat leaf blast (W_LB), but reductions in the percentage of affected spikelets or in the percentage of affected leaf area, respectively, were not observed. Results from chapters 2 and 3 suggested that the AMPs Ace-AMP1, WD, ARACIN1, and Zeamatin did not have an *in planta* antifungal activity against the fungi tested in this study, and then, they did not confer resistance to either FHB or WB. Nevertheless, it is important to consider that the synthesis of these AMPs in transgenic plants at the protein level was not evaluated, and the

expression of the transgenes was only confirmed up to the messenger RNA (mRNA) level. To evaluate if the transgenic plants are synthesizing biologically active peptides, crude protein extracts could be used in *in vitro* antifungal activity tests. The activity of protein extracts on the growth of *F. graminearum* and MoT could be tested by microplate inhibition assays (Broekaert et al., 1990; Cavallarin et al. 1998), and other fungal species, in which the antifungal activity of these AMPs was previously reported, could be used as controls. In addition, several authors have discussed the role of plant AMPs in the modulation of the defense response (Bolouri Moghaddam et al., 2016; Campos et al., 2018;). Roy-Barman et al. (2006) reported that expression of *Ace-AMP1* in transgenic wheat resulted in changes in the expression of defense-related genes and accumulation of salicylic acid. Although there is no information on the role of the other three AMPs in modulating the basal response, possible changes in the expression levels of defense-related genes would be an indirect evidence of the biological activity of these peptides.

In chapter 3, the introduction of a rice resistance gene into wheat, to confer resistance to WB was also described. Contrasting with the limited knowledge about the interaction MoT - wheat, the interaction between rice and the causal agent of rice blast, *M. oryzae Oryza* pathotype (MoO), has been widely characterized. Several rice resistance (*R*) genes, and MoO avirulence (*AVR*) genes have been described, and some of them cloned (reviewed by Wang et al., 2017; Kalia & Rathour, 2019). In this study, the presence/absence of genes homologs to the MoO effector genes was characterized in a group of South American MoT isolates, in order to identify genes with high prevalence in populations and then to select cognate rice *R* genes that can potentially confer resistance to MoT. Among 22 effector genes tested, four AVR genes, *AVR-Piz-t*, *AVR-Pi9*, *AVR-Pi54* and *ACE1*, were present in more than 94% of the isolates. Wheat

transgenic plants expressing the rice *R* gene *Piz-t* were challenged with MoT isolate T-25, and two lines showed a reduction in the percentage of leaf area affected, but no line showed a decrease in the percentage of spikelets affected. In rice, the interaction between *Piz-t* and *AVR-Piz-t* is not direct, and the resistance response involves at least three different host proteins (Park et al., 2012; Park et al., 2016; Wang et al., 2016). The low resistance response observed in wheat transgenic plants expressing *Piz-t* could result from the absence of homologous proteins that mediate the resistance in wheat. Other *AVR* genes such as *AVR-Pi54* and *AVR-Pi9* were also found in high proportion in the MoT group analyzed, and the corresponding cognate genes, *Pi54* and *Pi-9* would be good candidates to be incorporated in wheat. In addition, it has been established that the interaction between *Pi54* and *AVR-Pi54* is direct (Ray et al., 2016), which would facilitate recognition and resistance response. Before exploring this alternative, it is necessary to evaluate if the *AVR-Pi9* and *AVR-Pi54* alleles, present in the MoT population, are functional or not. This can be done by transforming a rice pathogen (MoO) with the MoT allele, and evaluating the interaction with a rice material carrying the corresponding resistance gene.

In chapter 4, the second approach used in this study, the implementation of CRISPR/Cas9 to edit the wheat translation initiation factors *eIF(iso)4E-2* and *eIF4G*, is discussed. The proteins eIF(iso)4E-2 and eIF4G are components of the translation initiation complexes eIF4F and eIF(iso)4F, and they are recruited for several families of viruses to translate their genome (Safaçon, 2015), including the family *Potyviridae* to which *Wheat streak mosaic virus* (WSMV) belongs. Virus resistance conferred by the loss of interaction between these factors and viral RNA has been observed in natural mutants (recessive resistance) and it has been replicated in several plant species by using silencing and knockout of the genes encoding the eIF4F and eIF(iso)4F factors (Schmitt-Keichinger, 2019). The silencing of the wheat *eIF(iso)4E-2* and

eIF4G genes using RNA interference (RNAi), resulted in enhanced resistance to several viruses, including WSM, *Triticum mosaic virus* (TriMV), and *Soil-borne wheat mosaic virus* (SbWMV) (Rupp et al., 2019). A CRISPR/Cas9 editing approach was implemented to reproduce the resistance reported by Rupp et al. (2019), but with the aim of recovering transgene-free resistant plants. Plants with mutations in both targeted genes were recovered, and two plants had mutations in four and six alleles of *eIF(iso)4E-2* and *eIF4G*, respectively. Reductions in the level of expression of the targeted genes were confirmed in the homozygous progeny of these two plants, but upon infection with WSMV isolate 'Sidney 81', plants developed characteristic symptoms and differences in virus accumulation were not detected when compared to wild-type plants. These unexpected results suggested that WSMV may be using different isoforms to complete the translation of its genome or that knockout mutations induced WSMV to hijack other isoforms, as has been suggested by some authors (Bastet et al., 2017; Gauffier et al., 2016). A better understanding of the factors interacting with WSMV is crucial to develop proper management strategies; likewise, the design of variants of the factors by inducing point-mutations, instead of loss-of function mutations, should be explored.

Although the results obtained in this study were not the expected outcome, the implementation of biotechnological tools to incorporate resistance to plant diseases has enormous potential, particularly the CRISPR-based editing approaches. The recent publication of Su et al. (2019), where CRISPR/Cas9 was used to generate mutations in the wheat endogenous gene *TaHRC*, enhancing resistance to FHB, is an exceptional example. CRISPR-based editing approaches offer several alternatives to improve or incorporate resistance, the edition of well characterized endogenous susceptibility genes can be expanded to other diseases like tan spot and Septoria nodorum blotch, caused by *Pyrenophora tritici-repentis* (*Ptr*) and

Parastagonospora nodorum (*Pn*, formerly *Stagonospora nodorum*), respectively. Both necrotrophic pathogens produce host selective toxins, ToxA, ToxB and ToxC produced by *Ptr,* and ToxA, Tox1 and Tox3, produced by *Pn*, which are needed to cause disease (Figueroa et al., 2018). Genes involved in sensitivity to the toxins would be ideal targets to induce CRISPR-based mutations, and several wheat genes responsible for the sensitivity have been described, for example, *Tsn1* is involved in the sensitivity to ToxA, a toxin produced by both pathogens, although the proteins do not interact directly (Faris et al., 2010); *Snn*1 interacts directly with Tox1 (Shi et al., 2016); *Tsc2* and *Tsc1* which interact with ToxB and ToxC, respectively (Effertz et al., 2002; Friesen and Faris, 2004). *Ptr* and *Pn* races are differentiated by the expression of one or several toxins, and host sensitivity to a single toxin is enough to induce necrosis (Ciuffetti et al., 2010). Durable and effective resistance to this kind of pathogens should involve the simultaneous editing of several susceptibility genes, and then some of the multiplex genome editing tools developed for wheat (Wang et al., 2016) should be implemented.

Creation of allelic variants by targeted single-nucleotide substitutions or base-editing is another alternative to incorporate resistance to wheat pathogens. The successful use of a Cas9 variant fused with a cytidine deaminase to induce C to T substitutions in the wheat *TaLOX2* gene was already described by Zong et al. (2017). Likewise, the efficacy to generate resistance to diseases was reported by Bastet et al. (2019), who showed that mutations induced by a CRISPR-Cas9 cytidine deaminase in the wild-type *eIF4E* gene of *Arabidopsis* conferred resistance to *Clover yellow vein virus* (ClYVV). The creation of allelic variants could have some advantages over the knockout or loss-of-function mutations, for example, (i) increased durability of the resistance, since pathogens can overcome the “knockout resistance” by adapting to other host proteins; (ii) wide range of resistance, because resistance to different races can be designed; and

(iii) several genes/alleles can be edited simultaneously without cause a deleterious effect on plants because proteins are still functional.

Together with knockout mutations and targeted single-nucleotide substitutions, introduction (knock-in) and stacking of resistance genes from wild relatives, or allele replacements can be facilitated and accelerated by CRISPR, reducing the linkage drag. Although the repair of double-strand breaks by the homologous recombination pathway is inefficient, the frequency of gene-targeting is improved when a high copy number of repair templates and nucleases are provided. Gil-Humanes et al. (2017) reported increased frequencies in gene-targeting when they used *Wheat dwarf virus*-derived DNA replicons to deliver both the CRISPR/Cas reagents and the repair template into the cell.

With the rapid development of CRISPR-Cas nucleases and base editors for plant genome editing (reviewed by Gürel et al., 2020; and Satheesh et al., 2019), and the possibility of generating transgene-free edited wheat plants by using RNA or ribonucleoproteins complexes (Liang et al., 2017; Zhang et al., 2016), this editing system has the potential to be tailored to multiple applications. In addition, wheat elite materials could be directly edited, without the need to go through crosses with non-commercial materials used routinely in transformation. Hamada et al. (2018) reported the direct delivery of DNA plasmids expressing the CRISPR/Cas9 components to shoot apical meristems by biolistic, with a mutation efficiency of 5.2%, and 1.4% of mutation heritability. This method would allow recovering edited plants without going through tissue culture, therefore potentially several wheat materials could be transformed/edited. The CRISPR- based editing toolkit expands constantly, but the main bottleneck is the selection of appropriate genes, and it is there that a better understanding of the interaction between wheat and pathogens is critical. Undoubtedly, this tool will help generate crops with improved traits,

including disease resistance, which will ultimately contribute to crop sustainability and food security.

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