RNA INTERFERENCE MEDIATED VIRUS RESISTANCE IN TRANSGENIC WHEAT

by

JESSICA LYNN SHOUP RUPP

B.S., Pittsburg State University, 2009

AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

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Department of Plant Pathology
College of Agriculture

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Abstract

Wheat streak mosaic virus (WSMV) and *Triticum mosaic virus* (TriMV) are two viruses affecting wheat in the Great Plains region of the United States. Genetic resistance is severely limited, requiring management methods focusing on the deployment of resistant varieties and various cultural practices. Evaluation of resistance is complicated by the lack of a standard rating scale. The objective of this work was to develop new avenues to mitigate these challenges. A standardized virus symptom rating scale was developed using historical Kansas rating scales, and validated using multiple wheat populations. Two independent RNA interference (RNAi) expression vectors targeting portions of viral coat protein (CP) of WSMV and TriMV were previously transformed into wheat. T_2 plants and beyond were evaluated using PCR, reverse transcription-PCR and bioassays in which plants were challenged with their respective virus. These lines were evaluated for resistance through the T_6 generation. Crosses were made with the susceptible winter wheat cultivars, 'Overley' and 'Karl 92.' Real-time PCR results show viral titer was up to 20-fold lower in the T_6 transgenic lines, the F_1, and the BC_1F_1 compared to control plants. This provides evidence that this RNAi silencing method is stable in wheat over multiple generations. WSMV and TriMV use host eukaryotic initiation factors (eIF) in order to facilitate replication of their genomes. Previously created RNAi expression vectors were derived from the sequences of the wheat genes eIF(iso)4E-2 and eIF4G. Evaluation of these lines began in the T_1 generation. Resistance has been demonstrated in three lines of eIF(iso)4E-2 and four lines of eIF4G, derived by single seed descent. T_6 progeny co-infected with WSMV and TriMV continue to be resistant. Crosses have been performed with the winter wheat 'Karl 92' and three Kansas elite lines, KS030887K-6, KS09H19-2-3, and KS10HW78-1-1. RNAi construct effectiveness was evaluated using real-time PCR. Results show up to 18-fold

reduction in viral titer in the transgenic lines, the F_1, and the BC_1F_1 in comparison to control plants. This research provides the first evidence that a single host transgene can provide resistance to multiple viruses and has great potential benefits to both breeders and producers.

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Dedication

To my late father, Dr. Galen L. Rupp, Ph.D., who always taught me that you can never go wrong when you do what you love and that you can, in fact, survive a Ph.D. experience.

Chapter One - Introduction

Wheat (*Triticum aestivum* L.) is considered a staple crop around the globe and has tremendous economic importance (Slade, 2004). Wheat currently occupies more land than any other crop, and its' value for world trade is greater than all other crops combined (Curtis *et al*., 2002). In 2013, 220 million hectares were harvested, which resulted in 716 million metric tonnes of grain (FAO, 2015). Currently, the USA is the third largest producer of wheat. This equates to 63 million acres planted and 2500 million bushels harvested (USDA-ARS, 2012). There are over 240,000 farms in the United States that produce wheat and nearly half of this amount is exported into the global economy. Sixty-five percent of wheat produced comes from the Great Plains region. Kansas produces 20% of the total domestic production and in 2013, the state produced 321 million bushels of wheat, which accounted for $2 billion for the Kansas economy (USDA Crop Production 2014 Summary, National Agricultural Statistics Service).

Wheat was first cultivated approximately 10,000 years ago. The earliest known cultivated forms were einkorn wheat, (*Triticum monococcum*) which has a diploid genome (AA), and emmer wheat, (*Triticum turgidum* subspecies (ssp.) *dicoccoides*) which has a tetraploid genome (AABB). The origin of the (BB) genome is unknown, but may have originated from the section *Sitopsis*, which is in the same section as *Aegilop*s *speltoides* (Brenchely *et al*., 2012). These plants are hypothesized to have originated in southeastern Turkey, in the fertile crescent (Heun *et al*., 1997; Feldman, 2001; Dubcovsky and Dvorak, 2007). Hexaploid common wheat, *Triticum aestivum* (AABBDD), originated approximately 9,000 years ago when a hybridization occurred between emmer wheat and goatgrass, *Aegilops tauchii* (DD) (Feldman, 2001). It is suspected that this hybridization occurred multiple times and early farmers selected it for its

superior grain (Dubcovsky and Dvorak, 2007). Wheat now provides 1/5 of all the calories consumed by humans (FAO, 2006). Common wheat accounts for 95% of wheat grown, which is destined for bread and other baked goods. The remaining 5% is devoted to durum wheat, which is used for making pasta. The other types of wheat, including spelt and emmer, are considered of minor importance to food provisions (Zohary *et al*., 2000).

Wheat endures an onslaught of abiotic and biotic stresses. These can include drought, salt, heat, bacterial and fungal pathogens, and nematodes. Wheat crops also endure biotic stress caused by viruses. The 'wheat streak mosaic (WSM) complex' is a disease that can be caused by up to three distinct viruses: *Wheat streak mosaic virus* (WSMV), Wheat mosaic virus (WMoV, formerly called High Plains virus, HPV), and *Triticum mosaic virus* (TriMV). WSM causes annual yield losses of approximately 2 to 5 % in the Great Plains region (Appel *et al.*, 2007; Shahwan and Hill, 1984; Wiese, 1987). Over the last two decades WSM has been the second most important wheat disease in terms of yield loss in Kansas (Appel *et al*., 2007).

WSM symptoms are characterized by a yellow leaf streaking, a stippling pattern, stunting, head sterility, low seed test weights, and poor tillering (Fig. 1.1) (Murray *et al.*, 2005). Early infection can be confused with nutrient deficiency, drought, root rots, and early stages of fungal foliar diseases. Symptoms commonly appear first on the edge of the field as the virus and vector, the wheat curl mite (Fig. 1.2) (WCM, *Aceria tosichella* Keifer), moves into the crop from plants in ditches and infected crops that are adjacent or upwind (Figure 1.3) (Slykhuis, 1955). In general, early fall infection by WSMV in winter wheat causes the most significant damage to yield and provides the greatest amount of inoculum for spread in the spring (McMullen and Waldstein, 2010; Hunger, 2004; Thomas and Hein, 2003). Fall infection is difficult to diagnose because the symptoms may not be observed until temperatures warm up in the spring. The

WCM transmits WSMV through feeding on leaf tissue. Mites use their stylets to penetrate into the leaf mesophyll where the virus is deposited into the plant (Sabelis and Bruin, 1996). The virus then travels to the roots where it travels along with nutrients back into the upper leaves, creating a systemic infection (Hipper *et al*., 2013).

First discovered in Nebraska in 1922, WSMV is one of the most destructive viral diseases in wheat (Makkouk and Kumari, 1997) and is an important virus in small grain production systems distributed worldwide (Wiese, 1987). In 2008, WSMV was the greatest disease pressure in the state of Missouri. In Montana, statewide outbreaks of WSMV have been recorded in 1964, 1981, 1993, and 1994 (Bamford *et al.*, 1996; Burrows *et al.*, 2009). Yield loss in individual fields due to WSMV can approach 100% (McNeil *et al.*, 1996; Riesselman, 1993, French and Stenger, 2004) if early infection occurs on susceptible varieties. Depending on the variety, 25% to 80% yield loss has been recorded in Montana (Riesselman and Carlson, 1994) and 50.2 to 91.4% in winter wheat varieties in Colorado (Shahwan and Hill, 1984). In 1988, yield loss on spring wheat varieties due to WSMV infection in North Dakota was estimated at 31.9 to 98.7% (Edwards and McMullen, 1988). In Kansas, annual losses caused by WSMV are estimated near 2.5%, which causes around a $10 million yearly losses, but can account for over one-half of the disease losses (Bockus *et al*., 2001). Crop losses in Kansas due to WSMV in 1976 approached 13% (Lengkeck, 1979), and in the 1988, epidemic losses up to $130 million were reported (Sim *et al*., 1988). In 2006, Kansas growers suffered WSMV losses of $109 million (Kansas Wheat Commission, personal communication), with small scale epidemics occurring in both 2011 and 2012. While the main crop host is wheat, WSMV can also infect other cereals such as barley, millet, maize, sorghum, oat, triticale and many other weed species (Seifers *et al*., 1996; Sill and Connin, 1953).

WSMV is a member of the genus *Tritimovirus,* of the family *Potyviridae* (Stenger *et al*., 1998). WSMV is transmitted by the WCM in a semi-persistent manner (Seifers *et al*., 2006). WSMV is a non-enveloped, flexuous rod-shaped virus, approximately 700 nm in length, 11-15nm wide and consisting of 9,384 bases (Urcuqui-Inchima *et al*., 2001). It is a positive sense, single-stranded RNA. The viral RNA is translated as an uninterrupted protein (Fig 1.4), encoding 3035 amino acids (Stenger *et al*., 1998). Post-translational polyprotein processing occurs (Hull, 2002), during which the virus undergoes self-cleavage by three encoded proteases to form ten functional proteins. P1 is a trypsin-like serine proteinase at the amino terminal end of the polyprotein with *cis* auto-catalytic activity. NIa acts in *cis* and *trans*: HC-Pro is a conserved carboxy-terminal cysteine proteinase domain acting in *cis* to cleave the HC-Pro/P3 junction of the viral polyprotein (Stenger *et al*., 2006; Urcuqui-Inchima *et al*., 2001). Other proteins include the coat protein (CP), the nuclear inclusion protein B (NIb), the cylindrical inclusion protein (CI), protease 1 and 3 (P1, and P3 respectively), 6K1, 6K2, and the 5' viral protein genome linked, (VPg) and a 3'polyadenylated tail (Braake, 1971; Siaw *et al.,* 1985; Fauquet et al., 2005).

The primary function of the CP is to encapsidate the viral RNA (Shukla and Ward, 1989). Approximately 2000 copies of the CP surround the genome (Stenger *et al*., 1998). The CP has been shown to be involved in aphid transmission, cell-to-cell and systemic movement, encapsidation of the viral RNA, and in the regulation of viral RNA amplification (Urcuqui-Inchima *et al*., 2001). It can be divided into three domains. The C-terminal and the N-terminal domain are exposed on the surface of the particle. The N-terminal domain contains the major virus specific epitopes involved in systemic movement (Urcuqui-Inchima *et al*., 2001). The central domain has been found to be highly conserved and is believed to play a role in cell-to-cell

movement and virus encapsidation (Dolja *et a*l., 1994). The CP N-terminal domain of most potyviruses contains a DAG motif, which is highly conserved in the aphid-transmissable members of the family (Harrison and Robinson, 1988). WSMV does not contain this motif; suggesting a different mechanism for virus transmission of the *Tritmoviruses* (Stenger *et al*., 2005). The CP plays a role in cell-to-cell movement, as was confirmed with deletions of the N-terminal region which caused an impairment of cell-to-cell movement (Dolja *et al*., 1994). It has been found that the N-terminal domain of the WSMV CP functions in both host and strain-specific long distance movement (Tatineni *et al*., 2011). The C-terminus has been found to play roles in differential infection of wheat through host-specific long distance transport (Tatineni and French, 2014).

HC-Pro has been shown to be a multifunctional protein involved with aphid transmission and interactions with virions (Maia *et al*., 1996). HC-Pro can also be divided into three regions, the C-terminal, the N-terminal and the central portion. HC-Pro has proven to be a transmission determinant in aphids, dictating the degree to which certain aphids retain the virion within their stylets (Wang *et al*., 1998). It has also been shown to be involved in viral amplification, systemic movement (Cronin *et al*., 1995), and to have interactions with nucleic acids, including affinity to RNA (Maia and Bernardi 1996). Perhaps one of the most important roles of HC-Pro is to act as a silencing suppressor of post-transcriptional gene silencing (Anandalakshimi *et al*., 1998; Kasschau and Carrington, 1998). HC-Pro may also interfere with the activity of a plant host defense system that normally limits virus accumulation (Pruss *et al*., 1997).

WSMV NIa is one of three virus-encoded proteinases (Shahabuddin *et al.*, 1988; Murphy *et al.*, 1990). NIa is the major proteinase of potyviruses and is responsible for proteolytic cleavage conserved sequences located along the polyprotein (Carrington and Dougherty, 1987,

1988; Carrington *et al.*, 1988; Dougherty *et al.*, 1988). NIa has two domains: the N-terminal VPg domain and the C terminal proteinase domain, NIa-pro. NIa-pro processes both in *cis* and in *trans* to produce functional products. NIa processing occurs at different rates, efficiencies and sites in a regulated process (Urcuqui-Inchima *et al*., 2001). NIa-VPg has been shown to be involved in genome amplification and host specificity. The protein product, VPg, is attached covalently to the tip of the viral RNA and has been shown to mimic a methyl-7-G cap of mRNA directly interacting with the cap binding proteins eIF4E and eIF(iso)4E (Whitmann *et al*., 1997; Khan *et al*., 2006). This leads to the initiation of translation of the viral genome (Leonard, *et al*., 2000; Thivierge *et al*., 2005).

NIb is the RNA-dependent RNA polymerase (RdRp). NIb has been shown to interact with the NIa-VPg (Hong *et al*., 1995; Fellers *et al*., 1998) or NIa-Pro (Li *et al*., 1997; Daros *et al*., 1999). These proteins colocalize in inclusion bodies found in the nucleolus and the cytoplasm. NIb contains two nuclear localization signals (NLS) (Li *et al*., 1997) and has been hypothesized to be involved in controlling the transport of the protein to the nucleus, however, its function there remains unknown. There is also evidence of interaction between NIb and the host poly(A) binding protein, however, this interaction remains unclear (Wang *et al*., 2000).

The CI protein has NTP binding, NTPase, RNA binding, and RNA helicase functions. The protein belongs to the 'super family 2' of viral helicases, characterized by seven conserved segments (Kadare and Haenni, 1997). The CI has also been implicated in cell-to-cell movement (Carrington *et al*., 1998). The function of 6K1 remains mostly unknown. It has been hypothesized that it is involved as a pathogenicity factor (Saenez *et al*., 2000). 6K2 is known to be required for replication and serves as an anchor for the viral replication complex at the endoplasmic reticulum (Schaad *et al*., 1997).

P1, found on the N-terminal gene of the potyvirus genome encodes a proteinase which allows self-cleavage from the polyprotein (Riechmann *et al*., 1992). While P1 is not required for viral infectivity, it does enhance amplification and movement of the virus. However, cleavage at the boundary between P1 and HC-Pro is essential for viability. It was found that P1 cleaves in *trans* and stimulates genome amplification (Klein *et al*., 1994; Verchot and Carrington, 1995a,b). RNA binding activity has been reported for P1 (Brantley and Hunt, 1993) and it has been shown that the protein had the same affinity for dsRNA and single-stranded RNA. This non-specific RNA binding has been attributed to viral movement (Arbatova *et al*., 1998). The P1/HC-Pro fusion carries the potential of a broad pathogenicity enhancer, which can play a role in suppression of host defense and suppression of posttranscriptional gene silencing (Kasschau and Carrington, 1998).

Much of the function of the protein P3 remains a mystery. It has been found to contain the fewest sequence homologies among the *Potyviridae*, as well as a distinct lack of easily identifiable elements (Aleman-Verdaguer *et al*., 1997). Despite often conflicting results in experiments, it is hypothesized that P3 may be involved in virus amplification (Klein *et al*., 1997). P3, 6K2 and 6K1 have no RNA binding activity (Merits *et al*., 1998). Therefore, its participation in replication is believed to occur through its interaction with CI (Rodriguez-Cerezo *et al*., 1993). There is also evidence that P3 is involved in pathogenicity (Riechmann *et al*., 1995; Saenz *et al*., 2000). P3 contains a short open reading frame (ORF), named *pipo* for “pretty interesting potyviral open reading frame” embedded within the gene that is translated in a +2 reading frame by ribosomal frameshifting to -1 or transcriptional slippage (Chung *et al*., 2008). PIPO may have roles in replication, systemic silencing, or movement (Vijayapalani *et al*., 2012).

Triticum mosaic virus (TriMV), a relatively new virus discovered in Kansas in 2006, is a member of the *Potyvirus* family (Seifers *et al*., 2008), and the type member of the *Poacevirus* (Tatineni *et al*., 2009). TriMV was discovered infecting a WSMV-resistant wheat cultivar 'RonL' and the symptoms were identical to those caused by WSMV. TriMV is a distinct member of the *Potyvirus* family, and although the genome arrangement is identical to WSMV, amino acid sequence of this protein exhibited 49% similarity to a different potyvirus, *Sugarcane streak mosaic virus* (SCSMV) (Tatineni *et al*., 2009). The virus is vectored by the WCM, and virus transmission can occur individually or together with WSMV (Seifers *et al*., 2009). TriMV can also be mechanically transmitted. Its symptoms include yellow-green mosaic patterns on the leaves, general yellowing, leaf curling and stunting (Price *et al*., 2010). Co-infection with the other wheat mosaic complex viruses can result in more severe infections, correlating with higher levels of viral titer (Tatineni *et al*., 2010). Crop losses due to TriMV alone remain uncertain.

Control strategies for WSMV and TriMV are the same and based on cultural practices due to the lack of resistance available in commercial cultivars. The first would be to limit the increase and spread of the vector population. Secondarily, eliminate the sources of inoculum, including volunteer wheat and grassy weeds that can act as a reservoir. Volunteer wheat poses an increased risk for fall infestations, since mites readily move to the new plants (Staples and Allington, 1956). Pesticide applications prove to be ineffective due the life habits of the WCM. Producers are also advised to avoid early planting, because this can often allow mite establishment, reproduction and infection of the crop (Wegulo *et al.,* 2007; Staples and Allington, 1956).

Genetic sources of resistance have been found in lines that contain the resistance gene *Wsm1*, which was transferred to wheat from intermediate wheat grass *Thinopyrum intermedium*

(Host) Barkworth & DR Dewey (Friebe *et al*., 1991). However, many derived lines have shown poor bread-making quality or agronomic properties. *Wsm2*, from an unknown source is present in the germplasm line CO960293-2 (Haley *et al*., 2002) and transferred the cultivars 'RonL' and 'Snowmass' (Seifers *et al*., 2007; Haley *et al.,* 2011). Both of these resistance sources are temperature sensitive: effective at 18°C, but breaking down as temperatures rise (Seifers *et al*., 2006, 2007). A third gene, *Wsm3* conferring resistance to WSMV and TriMV was found in wheat in a compensating wheat-*Th. intermedium* (Host) Barkworth & Dewey ditelosomic addition line. This Robertsonian translocation came from 7A#3L to the short arm of chromosome 7B *Wsm3 is* resistant to both WSMV and TriMV at low temperatures of 18°C and temperature sensitive to TriMV at 24°C (Lui *et al*., 2011). Minor gene resistance or tolerance has been found in hexaploid bread wheat (Rahman *et al*., 1974; Martin *et al*., 1976; Seifers and Martin 1988) as well as genes for resistance to WCM (Martin *et al*. 1984; Tatineni *et al*. 2010).

Viruses must interact with host genes in order to complete their lifecycle. Eukaryotic initiation factors (eIF) are key determinants of the interactions between plants and several RNA viruses (Robaglia and Caranta, 2006). Viruses depend on these host genes in order to complete their life cycle. Many viruses from multiple families use the eukaryotic initiation factor complex for cell-to-cell movement, translation, and replication. VPg and the eukaryotic initiation factors, eIF4E and eIF4G, are required for infection (Diaz- Pendon *et al.*, 2004). The eukaryotic initiation factors belong to the eIF4F complex, which recruits ribosomes and cellular mRNAs to initiate protein synthesis. eIF4F and eIF4E bind the cap structure of the 5' end of mRNAs. eIF4A, a RNA helicase, unwinds the mRNA 5' UTR to facilitate the ribosome binding. eIF4G, which is a scaffold protein, interacts with other translation machinery components (Gingras *et al*., 1999). Plants have a second eIF4F complex containing eIF(iso)4F, which contains the

isoforms eIF(iso)4E2 and eIF(iso)4G (Browning, 2004). Potyviruses need these eIF4E isoforms. Research has shown that the disruption of eIF(iso)4E gene in Arabidopsis results in resistance to *Turnip mosaic virus, Lettuce mosaic virus*, and *Plum pox virus* while disruption of eIF4E gene results in resistance to *Clover yellow vein virus* (Duprat *et al*., 2002, Sato *et al*., 2005, Decrocq *et al*., 2006). eIF4G is also involved in the cap-independent translation process of viruses (Nicaise *et al*., 2007). It has been reported that the VPg of *Turnip mosaic virus* interacts with, but does not disrupt, the translation initiation complex eIF(iso)4E-eIF(iso)4G. VPg was also found to interact with the eIF4(iso)4G. The VPg interaction decreased the affinity of the translation initiation machinery for capped mRNAs (Plante *et al*., 2004). VPg inhibits host protein synthesis at an early stage of the initiation complex formation through the inhibition of cap attachment to the initiation factor eIF4E (Grzela *et al*., 2006). *Tobacco etch virus* VPg has been shown to interact with eIF4E or eIF(iso)4E (Gallie *et al*., 2001) and have been shown to be a determining factor in plant host susceptibility (Estevan *et al*., 2014).

Recessive resistance has been found in mutant alleles of eukaryotic initiation factors to specific viruses, such as *Clover yellow vein virus* (ClYVV) and *Turnip mosaic virus* (TuMV) in Arabidopsis. ClYVV accumulated in leaves of mutant plants lacking eIF(iso)4E, but not in mutants lacking eIF4E. TuMV replicated in mutant plants lacking eIF4E, but not in mutants lacking eIF(iso)4E, indicating selective requirement of elements of the complex during potyviral infection (Sato *et al*., 2005). Evidence also suggested the requirement of eIF4G for the interaction of eIF4F and VPg for infection. The VPg protein was shown to be the avirulence factor for recessive resistance genes in various plants (Nicholas *et al*., 1997, Keller *et al*., 1998). Two genes have been found in barley, *rym4* and the allelic *rym5,* that seem to initiate recessive resistance to different strains of the *Barley yellow mosaic virus* (BaYMV) and *Barley mild*

mosaic virus (BaMMV). The gene product contains unique amino acid substitutions in comparison with the wild-type proteins and are found at the mRNA cap-binding site (Kanyuka *et al.*, 2005; Stein *et al*., 2005). Stable transformation of resistant genotypes with eIF4E from susceptible plants induced susceptibility to BaMMV (Stein *et al*., 2005).

RNA interference (RNAi) is a biologically conserved response of eukaryotes to the presence of double-stranded RNA. RNAi, also known as post-transcriptional gene silencing serves to regulate the expression of host genes, as well as mediate resistance to parasitic and pathogenic nucleic acids. The first clue leading to the discovery of the process of RNAi came from work by Napoli *et al*., (1990), which produced white centered petunias by an overexpression of the enzyme chalcone synthase. RNAi was first demonstrated by Fire *et al.*, (1998) while performing experiments with nematodes, discovering that the presence of dsRNA is responsible for producing interfering activity of normal gene function.

Dicer, a ribosome III-like enzyme, is responsible for processing dsRNA to approximately 21-27 nucleotides (nt) in length (Bernstein *et al*., 2001; Carmell *et al*., 2004). They remain double-stranded at this stage (Hamilton and Balcombe, 1999). The DCR-2/R2D2 complex binds to the small interfering RNA molecules (siRNAs) (Liu *et al*., 2003), then incorporating siRNAs into a multi-subunit complex called the RNA induced silencing complex (RISC). The siRNA is a guide for RISC to recognize target mRNAs that are complementary to the sequence and directs their cleavage as well (Hammond *et al*., 2002). The dsRNA undergoes ATP-dependent unwinding, then, with little to no ATP, RISC can cleave the target mRNA that is complementary to the guide strand (Nykanen *et al*., 2001). The signal can then be perpetuated by siRNA amplification from single-stranded RNA into dsRNA with the help of RNA-dependent RNA polymerase (RdRP) found in eukaryotes (Zamore and Haley 2005). These signals move

systemically through the plasmodesmata and the phloem (Huntvagner and Zamore, 2002). Another type of small RNA called microRNA (miRNA) plays important roles for developmental regulation and timing. miRNAs are generated by Dicer from short hairpin structures, miRNA precursors (pre-miRNA), that are derived from longer primary miRNA transcripts (pri-miRNA) (Brodersen and Voinnet 2006). Mature miRNAs are incorporated into RISC to guide mRNA degradation. RNA interference (RNAi) has recently emerged as a useful tool for discovering or validating gene functions. It has also been used for engineering specific suppressions for the expression of a desired gene.

While RNAi is a naturally occurring process recognized as a regulator of endogenous genes, it is also thought to be an ancient method of plant viral defense (Buker *et al*., 2007, Poogin *et al*., 2001, Mello and Conte 2004). The beginnings of RNAi mediated viral resistance took place in coat protein-mediated virus resistance as the coat protein (CP) gene of *Tobacco mosaic virus* (TMV) transformed into tobacco produced resistant plants (Powell-Abel *et al*., 1986; Beachy, 1999). During the 1980's, the resistance was thought to be caused by the presence of the protein, but current understanding of RNAi has elucidated that resistance was most likely caused by targeted degradation of the virus *in planta* via RNAi (Lindbo and Dougherty, 2005).

In 1993, Lindbo and Dougherty expressed the coat protein of the potyvirus, *Tobacco etch virus* (TEV) in a series of experiments using translational and non-translatable versions of the CP. Several lines expressing the non-translatable version of CP showed complete resistance to TEV. Conversely, the lines corresponding to CP translatable versions showed a systemic recovery phenotype in the new leaves. Resistant lines were susceptible to *Potato virus Y*, a closely related virus. This confirmed the specificity of the mechanism. Their results indicated

that the mechanism involved in the resistance response was mediated in a sequence specific manner at the cytoplasmic level (Linbdo *et al*., 1993).

The potential of oligodeoxynucleotides to act as antisense agents that inhibit viral replication in cell culture was discovered 1978 (Zamecnik and Stephenson, 1978) and used in multiple systems. Three different regions of the viral genome of *Cucumber mosaic virus* (CMV), used as antisense agents, were evaluated in transgenic lines. One plant line, accumulating an RNA complementary to a region encoding a protein required for replication, was resistant (Rezaian *et al*., 1998). Transgenic potato plants resistant to *Potato leaf roll virus* (PLRV) (Kawcuk *et al*., 1991) were observed when examining the accumulation of either sense or antisense transcripts of the PLRV CP. While the sense transcript was predicted to be translatable, the antisense was not, but researchers could not find detectable CP. Resistance to the geminivirus, *Tomato golden mosaic virus* was conferred by RNA complementary to an ORF encoding a replication protein (Bejarano *et al*., 1992). Fewer transgenic plants than control plants developed symptoms and the symptoms were of reduced severity. In this study, viral replication was reduced and a correlation was found between the levels of the RNA and the resistance. These studies have proven applicable to potyvirus and their host systems.

WSMV pathogen derived resistance was attempted using the entire potyviral *NIb* replicase gene and the CP to transform wheat. Researchers found both resistant plants and plants with delayed onset of disease (Sivamani *et al*., 2000; Sivamani *et al*., 2002). In an independent experiment, a full length CP was used again (Li *et al*., 2005). Resistance was found in some of the lines containing the transgene in the T_1 generation, however analyses of T_2 and T_3 displayed transgene loss or silencing and the corresponding susceptible phenotypes (Li *et al*., 2005).

The idea of using hairpin constructs to generate dsRNA began in the early 2000's. Hairpins are made using inverted repeats with a linker sequence in between. Hairpin efficiency was initially tested using *Nicotiana tabacum* plants transformed with a construct encoding the NIa-protease (NIa-Pro) gene sequence of *Potato virus Y* (PVY) in sense and antisense, flanking the 800-nucleotide spacer derived from the *uid*A (*gus*) gene (Smith *et al*., 2000). Their results indicated resistance in up to 65% of the plants. A hairpin-less double strand construct containing an intron as a spacer was tested and the spacer was spliced out during pre-RNA processing. The results from this experiment indicate 96% plant resistance. Further work by Eamens et al., (2008) revealed that an inverted repeat of the transgene produced a greater level of resistance.

A hairpin construct of *Mungbean yellow mosaic India virus* (MYMIV), a single stranded DNA geminivirus, was transformed in black chickpeas (Poogin *et al*., 2003). It was found that the plants showed a complete recovery from infection of MYMIV that lasted until senescence. Cassava expressing dsRNA of the *AC1* gene of the geminivirus, encoding a replication associated protein had a 98% reduction of infection compared to the control plants (Chellappan *et al*. 2004). In the past several years, researchers have confirmed the effectiveness of deploying RNAi strategy for controlling viruses from different crops such as tobacco (Kamachi *et al*., 2007), soybean (Tougou *et al*., 2006), tomato (Fuentes *et al*., 2006) and wheat (Fahim et al., 2010). Accumulated research has confirmed that both DNA and RNA plant viruses could be efficiently controlled in transgenic plants by inducing RNAi. The silencing signal can spread throughout the entire plant thereby providing systemic resistance instead of localized resistance (Coinnet *et al*., 1998).

Once it became clear that single hairpins worked to generate resistance to plant viruses, researchers then moved on to stacking the hairpins within one construct. By a single construct

with multiple hairpins, researchers hope to generate resistance to more than one virus. Multiple viral resistance was achieved by using a single transgene construct (Bucher *et al*., 2006). A chimeric cassette was made targeting four different tospoviruses: *Tomato spotted wilt virus* (TSWV), *Groundnut ringspot virus* (GRSV), *Watermelon silver mottle virus* (WSMoV) and *Tomato chlorotic spot virus* (TCSV). The sequences formed the hairpin structures, which then triggered RNAi. Resistance frequencies of over 80% could be obtained to the four tospoviruses in a mixed infection.

The first commercial sale of a transgenic crop with virus resistance took place in the US in 1995, with virus-resistant squash by Asgrow Co, Freedom II (Kalamazoo, MI, USA). *Papaya ringspot virus* resistant papaya is often credited with saving the Hawaiian papaya industry (Gonsalves, 2014). *Plum pox virus* resistant plum has also been approved for commercialization. A major concern stemming from this type of technology revolves around virus evolution. Fear of generating more virulent strains are of concern to scientists, producers and consumers. One of these fears is the phenomena of heteroencapsidation, which occurs when the coat protein of one virus encapsidates the viral genome of another. This has the capability to induce changes in pathogenicity, transmission and virulence (Fuchs and Gonsalves, 2004). While this phenomenon has been reported in the lab (Farinelli *et al*., 1992; Fuchs *et al*., 1999), no instances of virus resistant fruits and vegetables have been reported in field studies. Also, heteroencapsidation is not heritable for the virus, so these events are limited to one generation (Fuchs *et al*., 1999). Another possibility stems from virus recombination. Recombinant viruses have chimeric genomes originating from more than one source. However, research on several species including plums, squash, grapevine and papaya have yielded no recombination in relation to the transgenic plants (Capote *et al*., 2007). The durability of PRSV resistant papaya has been proven in the field

under challenges from many different virus strains (Fuchs and Gonsalves, 2004.) RNAi based virus has not officially been through the regulatory process, however its mechanism is the same as CP-mediated resistance. Due to this, little evidence exists that it will not be as durable as papaya or squash has proven to be.

The three sources of natural resistance to WSMV in wheat have proven to be temperature sensitive (Seifers *et al*., 2006, 2007). Recent epidemics of the WSM complex necessitate additional work in order to combat these viruses in the field. This must be completed quickly, without introducing traits with negative agronomic qualities. Recent work with transgenic plants expressing a hairpin RNA of the viral gene *NI*a have shown excellent resistance to the virus (Fahim *et al*., 2010). This type of transformation could add resistance to widely deployed cultivars without destroying positive agronomic traits. The hypothesis was that RNAi mediated silencing of viral genes or endogenous wheat genes involved in the viral life cycle could induce resistance in wheat to these viruses. A previous graduate student, Luisa Cruz developed expression vectors targeting WSMV CP (Cruz *et al*., 2014), TriMV CP, WSMV 6K2-NIa, WSMV CI, and two wheat endogenous genes, eIF(iso)4E-2 and eIF4G. Evaluation of WSMV CP and TriMV CP transgenic wheat lines were completed through the T_2 generation, while other lines were not. Full characterization of these lines could provide meaningful solutions to the challenges viruses present. As a continuation of this work, the objectives of my dissertation were to:

- characterize previously made RNAi hairpin constructs to determine resistance to viruses;
- characterize transgenic plants at the molecular level;
- perform bioassays through the T_6 generation;
- assemble T_6 generation families homozygous for transgene presence;

- cross transgenic lines into winter wheat adapted cultivars;
- evaluate transgenic lines and all crosses in bioassays;
- use qPCR to detect levels of viral titer and gene knockdown in transgenic lines and crosses;
- standardize the currently used virus rating scales to a single system based on phenotypic scores and levels of viral titer.

Figures

Figure 1.1: A wheat plant infected with *Wheat streak mosaic virus* (WSMV) confirmed by ELISA in the field in NE Kansas, 2011.

Scale bar = 10 μm, Photos: UNL

Figure 1.2: *Aceria tosichella* Keifer, the wheat curl mite on wheat leaves and viewed under Scanning electron microscope. Photos courtesy of University of Nebraska Extension Service www.unl.edu

Figure 1.3 Wheat field in NE Kansas in 2011 infected with WSMV from adjacent field. Symptoms can be seen to be greater in severity along the roadside, and decreasing towards the center of the field.

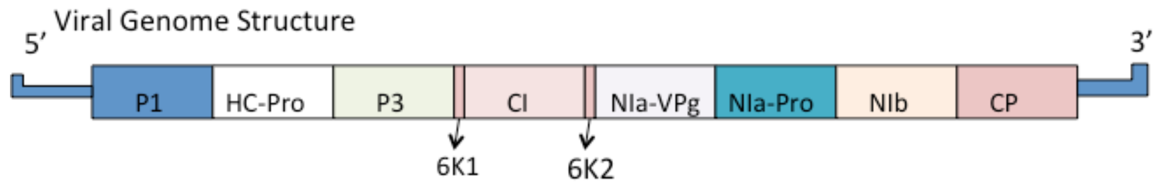

Figure 1.4 Viral genome structure of the Potyviruses *Wheat streak mosaic virus* (WSMV) and *Triticum mosaic virus* (TriMV)

Chapter Two - Stable resistance to *Wheat streak mosaic virus* in wheat mediated by RNAi

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Abstract

Wheat streak mosaic virus (WSMV) is one of the major wheat viruses found in the Great Plains of the USA. Cultural practices are the primary methods of disease management, though not fully effective. Although genetic resistance is available, it is temperature sensitive and is sometimes closely linked with traits having negative agronomic effects. Alternative approaches to viral resistance are clearly needed. RNA interference (RNAi) has been shown to play a role in viral defense response and has been successfully used as a biotechnological tool to preprogram viral resistance in transgenic plants. In this work, a portion of the coat protein of WSMV was used as a hairpin construct and was co-transformed with pAHC20-*bar* to elicit viral resistance. Eleven WSMV RNAi independent transgenic events were obtained. Thirteen T_1 lines were resistant as evident by the lack of viral RNA within the tissue. Beginning in the T_2 generation, single-plant lineages were selected, selfed, and evaluated for resistance and presence of the transgene until the T_5 generation. Families were then evaluated for the presence of the transgene, presence of the selectable marker, and WSMV resistance. Each of the lines in the T_5 generation were resistant to the virus. Generational selection has maintained expression of the transgene and resistance to WSMV.

Keywords: *Wheat streak mosaic virus* · Stable expression · RNAi · Transgenics

Introduction

Wheat is host to a wide range of viruses that are responsible for significant annual crop losses. *Wheat streak mosaic virus* (WSMV) and *Triticum mosaic virus* (TriMV) (Seifers *et al*., 2008; Burrows *et al*., 2009) are two widely distributed viruses with significant economic impact on wheat. The average annual yield loss caused by WSMV is estimated to be 2.5%. WSMV is transmitted semi-persistently by the wheat curl mite, Aceria tosichella Keifer, either as a single or mixed virus transmission (Slykhuis 1955; Seifers *et al*., 2009). Control strategies for WSMV include limiting spread of the vector population by controlling the mite and eradicating WSMV hosts such as volunteer wheat and grassy weeds. WSMV, first discovered in Nebraska in 1922, is a member of the *Potyviridae* family in the genus *Tritimovirus* (Stenger *et al*., 1998). Genetic resistance to WSMV infection is limited to three sources (Friebe *et al*., 1991, 1996, 2009; Haley *et al*., 2002; Lu *et al*., 2011), two of which are temperature sensitive (Seifers *et al*., 2006, 2007).

In plants, the posttranscriptional gene-silencing mechanism acts as a natural defense strategy against virus infection. RNA silencing allows cells to distinguish nonself messenger RNA (mRNA) and target these RNAs for degradation (Pooggin *et al*., 2001). The RNA interference (RNAi) process involves three basic features: induction by double-stranded RNA (dsRNA), processing of dsRNA into 21- to 25-nt small- interfering RNA (siRNA), and the inhibitory action of an effector complex that targets mRNA homologous to previously incorporated siRNA (Ruiz-Ferrer and Voinnet Olivier, 2007). DNA and RNA viruses, as well as transgenes that contain hairpin structures, can induce RNA silencing (Smith *et al*., 2000; Sivamani *et al*., 2000; Bucher et al. 2006; Fahim *et al*., 2010). Attempts to generate RNAi resistance to viruses in wheat have used full-length WSMV coat protein (CP; Sivamani *et al*.,

2000; Li *et al*., 2005), full-length NIb replicase (Sivamani *et al*., 2000), a hairpin construct of the NIa protease (Fahim *et al*., 2010), and a multi-target RNAi construct (Fahim *et al*., 2011), with varying levels of success. Full-length constructs provide resistance in early generations but the trans- gene is silenced in later generations (Anand *et al*., 2003b; Li *et al*., 2005) or the plants begin to show a milder resistance phenotype (Sivamani *et al*., 2000). Hairpin constructs have shown an increase in the efficiency of posttranscriptional gene silencing (Smith *et al*., 2000) compared to sense or antisense constructs alone. The objective of this work was to use RNAi hairpins containing a portion of sequence derived from WSMV CP as an approach for obtaining viral resistance and to determine the stability of the transgene in multiple generations.

Materials and Methods

Cloning and plasmid construction.

WSMV Sydney-81, obtained from Dr. Dallas Seifers (Kansas State University, Hays, KS), was maintained in the susceptible wheat cultivar 'Tomahawk,' released by AgriPro in 1990, and used as a template. mRNA was extracted by collecting 100 mg of symptomatic leaf tissue, grinding in liquid N_2, homogenizing in 1 ml of TRIZOL® reagent (Life Technologies, Carlsbad, CA), and incubated for 5 min at room temperature. Two hundred microliters of chloroform was added to the homogenate and the sample was vortexed for 30 s. Samples were centrifuged at 12,100×*g* in an Eppendorf 5415 C centrifuge at 4°C. Total RNA was precipitated using 500 µl 100% isopropyl alcohol, washed with 500 µl 75% EtOH, and resuspended in 40 µl diethyl pyrocarbonate (DEPC)-treated water. RNA was quantified using a NanoDrop spectrophotometer (NanoDrop Technologies, Rockland, DE). Reverse transcription (RT)-PCR was performed by denaturing 1 mg total RNA at 65°C for 15 min, then placing on ice for 5 min. The RT reaction included 5 mM $MgCl_2$, 1X reverse transcription buffer (10 mM Tris–HCl pH 9.0, 50 mM KCl,

0.1% Triton® X-100), 1 mM of each dNTP, 1 U/µl recombinant RNasin® ribonuclease inhibitor, 1 U AMV reverse transcriptase, and 0.5 µg random primers/µg RNA added (Promega, Madison, WI). Samples were incubated at room temperature for 10 min followed by incubation at 42°C for 1 h. Samples were heated at 95°C for 5 min to inactivate enzymes and placed on ice.

Primers were designed from the CP sequence of WSMV (GenBank accession no. AF057533; Stenger *et al*., 1998) using Primer Quest and OligoAnalyzer 3.0 (Integrated DNA Technologies, Coralville, IA; www.idtdna.com), and are listed in Table 2.1. CACC was added to the 5′ end of both forward primers for directional cloning of the PCR fragment into the entry vector pENTER-D/TOPO (Life Technologies) which were made containing a 386-bp WSMV CP fragment corresponding to nucleotides 8821–9207 of WSMV Sidney-81 (GenBank AF057533; Stenger *et al*., 1998). CP fragments were independently cloned into the pANDA-mini vector (Miki and Shimamoto 2004; Fig. 2.1) by means of homologous recombination via LR Clonase (Invitrogen, Carlsbad, CA). Plasmids were transformed into JM109 competent Escherichia coli cells (Promega) and purified using an E.Z. N.A. Plasmid Mini Kit (Omega Bio-Tek, Norcross, GA). Insert orientation was determined by Sanger sequencing (Kansas State University DNA Sequencing and Genotyping Facility).

Plant transformation and culture.

Immature seeds (10–14 d post anthesis) of the *Triticum aestivum* L. cultivar 'Bobwhite' were harvested from greenhouse-grown plants and surface sterilized with a solution of 20% v/v sodium hypochlorite (6%) and 0.04% v/v Tween-20. Excised embryos were cultured on callus induction medium CM4 (Zhou *et al*., 1995) in the dark at room temperature. After 1 wk, organized callus on the scutellum was selected for bombardment, placed on fresh medium, and air-dried for 1 h with the lids removed in a laminar flow hood to plasmolyze the cells. Wheat

transformation was performed through biolistic particle delivery as described by Anand *et al*. (2003a, b). Embryos were co- bombarded with the vector pAHC20 (Christensen and Quail, 1996) containing the ubiquitin promoter and the WSMV CP RNAi construct. Tissue culture media, selection, and culture were as described in Anand et al. (2003a). One hundred eighty embryos were used in each bombardment on five different occasions.

Transformant characterization

Putative T_0 transgenic plants were transferred to soil and tested for glufosinate resistance 1 wk after transplanting by brushing a 0.2% v/v Liberty (glufosinate) solution (AgroEvo USA, Wilmington, DE) onto one third of the surface area of a single leaf using a small paintbrush. DNA was isolated from putative glufosinate resistant plants with an absence of necrosis and tested by PCR for the presence of bar and CP constructs. Genomic DNA (gDNA) was isolated from 10-mg leaf tissue using an E.Z.N.A. Plant DNA Kit (Omega Bio-tek). gDNA was eluted with 40 µl pre-warmed water. For PCR, 200 ng gDNA was used per reaction.

A high-throughput DNA extraction method was used for the T_2 and subsequent generations of transgenic material (Allen *et al*., 2006). Leaf tissue (3–6 cm^2) was collected from each plant and placed in a 96-well microtiter plate. Tissue samples were stored at −80°C and then lyophilized for 2 d. Two stainless steel beads were added to each well, and plates were shaken for 5 min on a Fleming Gray model C paint shaker (Certified Technology Inc., Niagara Falls, NY). Six hundred microliters of lysis buffer (500 mM NaCl, 100 mM Tris, 50 mM EDTA, 5.0 mg sodium bisulfate, 0.1% ascorbic acid, 2% polyvinylpolypyrrolidone) was added to the powder and the plates were vortexed for 4 min. Plates were incubated for 1 h at 65°C and centrifuged at 3,000×*g* for 10 min. DNA was precipitated by centrifugation after the addition of

50 µl 10 mM NH_4OAc and 400 µl isopropanol to the supernatant. The resulting pellet was washed with 400 µl 70% EtOH and resuspended in 100 µl 1X TE buffer.

PCR analyses were carried out to identify the presence of bar from pAHC20 as well as the WSMV RNAi CP transgene. Each PCR reaction contained 10X ThermoPol Buffer (New England Biolabs, Ipswich, MA), 0.8 mM dNTPs, 25 pmol of each primer (Table 2.1), 1.25 U Taq DNA polymerase (New England Biolabs), and 200 ng template DNA. Amplification conditions were 5 min at 95°C; 30 cycles of 30 s at 95°C, 40 s at 60°C, and 60 s at 72°C; and a final extension at 72°C for 15 min. Seed from eight T_0 plants were analyzed for the presence and segregation of the insert using gus F1 and WSMV-R PCR primers (Table 2.1). Data from plants from the same event were pooled and tested using a chi-square test. For the Southern analysis, DNA digestion, membrane transfer, washing, and detection were as described in Faris *et al*., (2000).

Transgene expression was determined by extracting total RNA and deriving single-stranded complementary DNA (cDNA) by reverse transcription (catalog no. A3500, Promega). cDNA was used as a template for two PCR reactions. Expression of the *gus* linker was determined by using *gus* sense and antisense primers (Table 2.1) to amplify a 636-bp fragment. A second reaction determined the presence and replication of viral RNA by PCR of the cylindrical inclusion (CI) gene fragment of WSMV. Genomic DNA presence in the cDNA was determined using α-tubulin primers (Li *et al*., 2005).

Bioassays

Bioassays were carried out on plants at the three-leaf stage. Inoculum was prepared by macerating 1 g of fresh infected leaf tissue in 20 ml 0.02 M phosphate buffer, pH 7.2. After a passage through cheesecloth, the filtrate was added to a Devilbiss atomizer sprayer (Devilbiss,

Long Grove, IL) with 0.3 g carborundum powder. Inoculations were made using compressed air at 25–30 psi, on the adaxial surface of the second leaf until a small "water-soaked" spot was present. After inoculation, plants were incubated in a growth chamber with 16-h day/8-h night, 500 μE/m^2/s light intensity, at 19°C. Leaf samples were taken 14–21 d post-inoculation. Later generations were inoculated using the finger-rub technique by lightly dusting carborundum onto the second leaf and applying 40 μl of infected plant sap (100 mg of desiccated leaf tissue in 100 μl 0.02 M sodium phosphate buffer, pH 7.4) to the second or third leaf of 2-wk-old seedlings. The leaf was then pinched between the thumb and forefinger and the plant sap was pulled down the length of the leaf several times. Plants were inoculated again 10 d later to ensure infection. Virus presence was determined by indirect double antibody sandwich enzyme-linked immunosorbent assay (DAS- ELISA) according to the manufacturer's protocol (Agdia, Elkhart, IN). Samples from transgenic material, non-transgenic inoculated wheat, and mock-inoculated samples were used in the assays.

Results

Approximately 900 wheat callus explants were bombarded in five independent biolistic transformation experiments and 136 putative transgenic plants were regenerated (Fig. 2.2 A–D). After screening with the herbicide glufosinate, only 13 plants were identified as transgenic. Tillers from these T_0 plants were labeled alphabetically (A, B, C, etc.) because there was a chance that some of the tillers could be separate independent events or escapes. T_0 tillers E and F from event 34, tillers A and B of event 35, tillers A and B of event 110, one tiller from event 147, and one tiller from event 195 were found to have the WSMV CP hairpin construct (Fig. 2.3). Three tillers from event 479 appeared to have only a portion of the construct, as only the gus F1 and WSMV-R primer combination would amplify a band (not shown). Tiller 147D,

which emerged after glufosinate screening, did not contain bar and was later confirmed to be glufosinate sensitive. Although this tiller was an escape, its seeds were harvested and used as a control line that had passed through tissue culture.

T_1 generation

Seeds from T_0 tillers were harvested separately. All of the lines fit a 3:1 segregation ratio, indicating a single locus (Table 2.2). The T_1 lines were also subjected to virus inoculation. At 21 d after inoculation, samples from the youngest leaves were taken for molecular analysis and symptoms were recorded (Fig. 2.2 E, F). All of the plants, first selected for glufosinate resistance, except those from 147D, contained the transgene (Fig. 2.4). Symptoms were absent from plants 34E-6; 34F-2; 110A-6 110B-3, 110B-4, and 110B-8, and all but one of the plants from 195A. All of the plants from 110C, 147A, and 147D expressed WSMV symptoms. Viral RNA could be detected in plants with symptoms, including 195D-2, which had a detectable but faint band. With the exception of 195A2, which had symptoms despite having no detectable viral RNA, the lack of viral RNA was correlated with the lack of symptoms (Fig. 2.4). The negative-control nontransgenic plants showed 100% virus incidence, as did plants from line 147A.

$T_{2\text{-}5}$ analyses

Due to high levels of resistance to infection in the T_1 generation and the presence of a single transgene locus, progeny from T_1 plants 110B-4, 110B-8, 195A-20, and 195A- 17 were evaluated for transgene presence and virus resistance. The T_2 progeny of 110B-4 and 110B-8 all contained the transgene and were resistant to the virus (Fig. 2.5), though three lines of 110B-4 had slightly higher levels of virus detected via DAS-ELISA. Line 195A-20 had three out of ten plants with an increased level of virus as compared with negative controls. Line 195A-17 was

found to be segregating, as three of nine plants did not contain the transgene. Additionally, the highest level of CP antigen in this family appeared in one of the insert-positive plants. All five of the 'Bobwhite' controls displayed a susceptible phenotype.

Beginning with T_2 plants, single-plant selections were made, selfed, and progeny-tested for presence of the transgene and resistance to infection. Five of the T_3 families were found to be segregating for the transgene. Ten of the T_4 families had plants without the transgene and were susceptible (data not shown). Resistant plants were selected from the T_4 families and the progeny were again resistant. Presence of the *bar* gene, as determined by PCR, was evaluated at T_5, and it is still segregating in 13 of the families (Table 2.3). Southern analysis of the T_5 lines showed that 195A20:2 and 110B4:1 had multiple copies of the transgene (Fig. 2.6).

Discussion

Due to the limited genetic resources for resistance to WSMV, a transgenic approach was evaluated for resistance. A hairpin construct was introduced into wheat to target the WSMV CP. In theory, the RNA of the virus is targeted in the same manner as the RNA of the transgene and thus, makes the plant resistant to infection. Granted, this approach is not novel; however, here, we demonstrate stable transgene expression and continued resistance after multiple generational selection.

T_1 generation plants contained the CP hairpin insert and expressed the transgene in most cases (Fig. 2.4). However, after inoculation, only plants from lines 110 and 195 exhibited a significant number of resistant plants. Assuming that RNAi is eliciting plant viral defense, transgene expression levels could have been strong enough to elicit an effect but too low for detection. Another hypothesis to explain this phenomenon could directly involve an RNAi mechanism. The hairpin transgene, which contains dsRNA, is recognized by the Dicer-like

enzyme, inducing the RNAi mechanism (Helliwell and Waterhouse 2005). Dicer could have processed the transgene mRNA prior to viral inoculation, thus RNA would have become part of the RISC complex and target, preventing the detection of transgene mRNA.

Transgenic wheat lines developed by biolistic transformation can lose transgene expression within the first few generations. Plants expressing the full-length viral CP lost expression by the T_3 generation, which was attributed to the number of transgene copies generated from bombardment (Li *et al*., 2005). Different factors have been implicated in transgene silencing, such as interaction between multiple transgene copies, methylation and chromatin modification, or the interaction of these factors (Hammond *et al*.. 2000, 2001). It appears that lines derived from 195A have more copies that those from 110B (Fig. 2.6). Non-Mendelian ratios are evident in 195A17:2 and cannot be explained by segregation of a single dominant gene. It is possible that gene silencing is occurring; however, after several generations of selection, T_5 families appear to be completely resistant. Selection at each generation may eliminate lines with predisposition for non-RNAi silencing.

The presence of the *bar* gene was not a selection criteria from the T_2 through T_5 generations. However, the ratios of bar presence/absence were not consistent with Mendelian segregation (Table 2.3). Srivastava et al., (1996) showed a wheat line that physically lost the transgene in the T_3 generation. In *Arabidopsis thaliana*, the kanamycin resistance gene was lost (Feldmann *et al*., 1997) and rep-trap-ren transgenes for virus resistance in bean were also lost in later generations (Romano *et al*., 2005). This phenomenon is poorly understood, but might be caused by the genetic instability imposed by stress conditions during tissue culture (Risseeuw *et al.,* 1997). However, this does not explain the transgene loss observed in later generations. Intrachromosomal recombination could lead to the co-elimination of transgenes, where the

transgene integration site is a key factor in the elimination process (Fladung ,1999). An alternative explanation has attributed this phenomenon to mechanisms of genome defense (Srivastava *et al*., 1996).

Although the use of hairpin constructs for virus resistance is not unique, this is the first report of stable WSMV-resistant wheat produced by this method. In some lines, transgene silencing did occur; however, lines were produced that were stable through the T_5 generation by using multi-generational selection. Future work will determine if these transgenes maintain expression and provided resistance under field conditions and higher temperatures after being crossed into adapted varieties.

Acknowledgments: The authors would like to thank Dr. Jiarui Li, Dr. Jung Hoon Lee, Julie Essig, Sheila Stevens, Hyeonju Lee, Dehlia Burdan, Katie Neugebauer, and Zach Simon for their technical assistance and Dr. Robert Bowden for his comments. This work was supported by a grant from the Kansas Wheat Commission and USDA-ARS CRIS 5430- 21000-005D. This is a joint submission of the U.S. Department of Agriculture and Kansas Agricultural Experiment Station, Journal number 13-071-J.

Figures and Tables

Table 1. Primer sequences and descriptions of sequences used in this study

Primer name	Sequence (5′–3′)	Product size	Description
WSMV-F WSMV-R	CACCAATGCAGGCAAGGACAATGAGCAG TGCGTGTTCTCCCTCACATCATCT	386 bp	Amplification of WSMV CP fragment cloned in RNAi vector
gus F1 *gus* R2	CACGTAAGTCCGCATCTTCA GTATCAGTGTGCATGGCTGG	216 bp+GOI 154 bp+GOI	Used with the CP-specific primers to determine presence of GOI
gus Sense *gus* Antisense	CATGAAGATGCGGACTTCCG ATCCACGCCGTATTCGG	636 bp	RT-PCR primers to establish *gus* linker expression
BarAB-R UbiAB-F	CCTGCCTTCATACGCTATTTATTTGC CTTCAGCAGGTGGGTGTAGAGCGTG	500 bp	Amplification of Bar Gene (co-bombardment)
CI-F CI-R	TCCAGGAATGGGCGTGTGATGATA ACACTAGCATCTCTGCCGAGGTTT	256 bp	Used in RT-PCR to determine virus presence in transgenic material
Tub-F Tub-R	ATCTGTGCCTTGACCGTATCAGG GACATCAACATTCAGAGCACCATC	409 bp cDNA 500 bp gDNA	RT-PCR primers for internal control used to determine gDNA contamination

CP coat protein, *GOI* gene of interest

Table 2.1: Primer sequences and descriptions of sequences used in this study

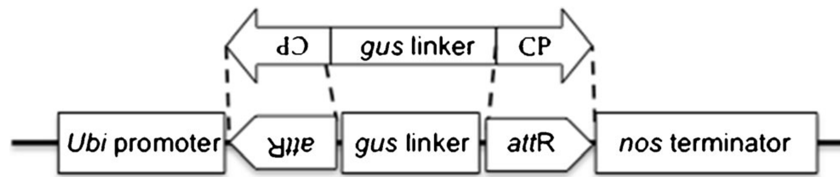


Figure 2.1: Schematic representation of WSMV CP RNAi vector construction. pANDA-mini (Miki and Shimamoto, 2004) vector with *att*R sites to allow for the homologous recombination insertion of inverted CP sequences flanking a *gus* linker. When expressed in wheat, the inverted CP sequences in the RNA form a hairpin due to Watson–Crick pairing and then are targeted by the Dicer system.

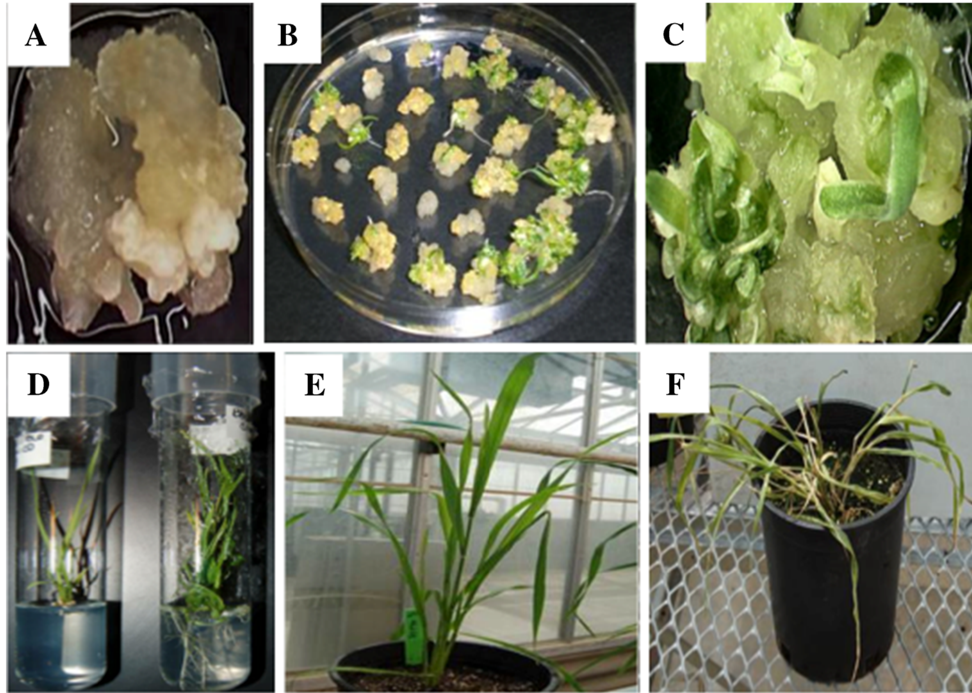


Figure 2.2: Tissue culture selection on glufosinate. Embryogenic callus formation (A), early shoot generation of selected calli (B), multiple shoot regeneration from a single callus (C), and root generation on shoots from selection (D). E, F Plants in the T_1 generation were inoculated with Wheat streak mosaic virus. At 21 d post-inoculation, these were rated as resistant (E) and susceptible (F).

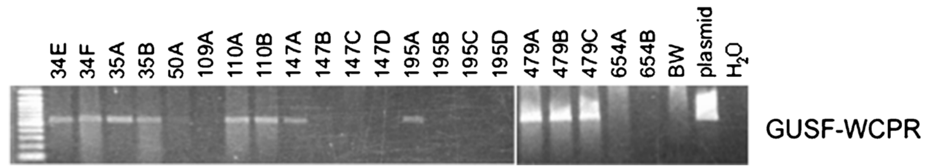


Figure 2.3: PCR analyses on gDNA of T_0 WSMV CP RNAi transgenic wheat. GUS forward (gus F1) and WSMV-R (WCPR) primers were used on genomic DNA. Each plant represented was resistant to application of glufosinate. Controls were non-transgenic Bobwhite (BW), plasmid DNA for PCR positive control, and water only (H_2O).

Line/tiller	Total	Ratio tested	Observed		Expected		Chi-square		Sum	*p* value
			+	–	+	–	+	–		
35A, B	15	3:1	10	5	11.25	3.75	0.14	0.42	0.56	0.4561
110A, B	13	3:1	9	4	9.75	3.25	0.06	0.17	0.23	0.6310
147A	15	3:1	13	2	11.25	3.75	0.27	0.82	1.09	0.2967
195A	22	3:1	17	5	16.5	5.5	0.02	0.05	0.06	0.8055

Table 2.2: Segregation analyses of T_1 generation for WSMV hairpin construct

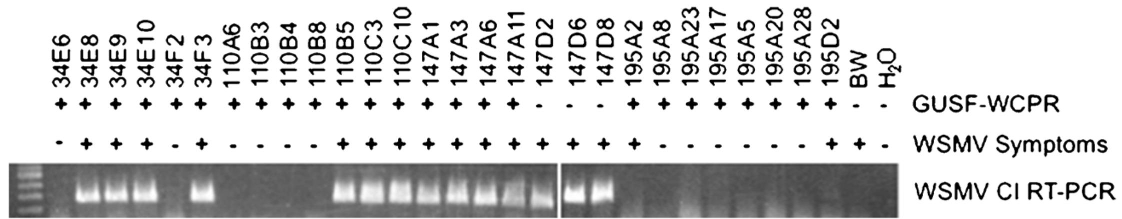


Figure 2.4: Analysis of T_1 plants derived from T_0 transgenic individuals. Plants were PCR-tested for the presence of the transgene by using *gus* F1 and WSMV-R (WCPR) primers. Symptoms were visually rated and the presence of the virus was determined by RT-PCR using primers for WSMV cylindrical inclusion (CI) protein. Line 147D was used as a tissue culture control. Bobwhite (BW) was used as a non-transgenic control.

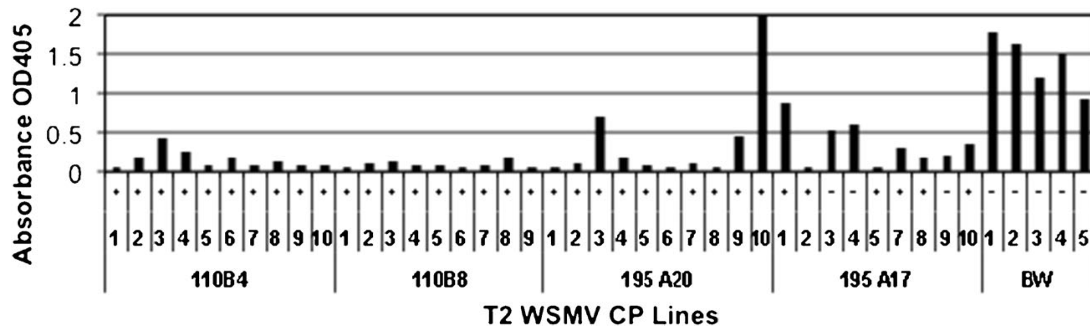


Figure 2.5: DAS-ELISA detection of *Wheat streak mosaic virus* coat protein (WSMVCP) antigen in in T_2 transgenic lines. Individual plants were assayed for the presence of the transgene (+/−) and CP antigen. Bobwhite (BW) was used as a non-transgenic control.

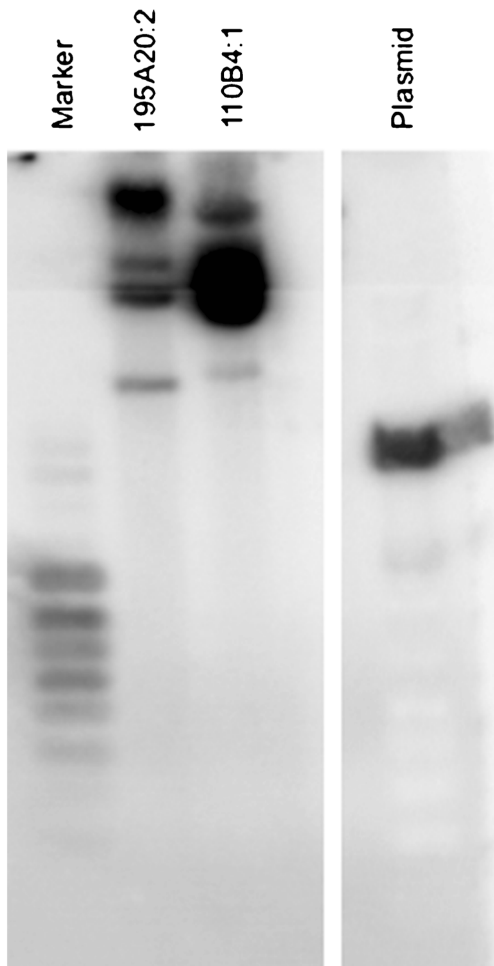


Figure 2.6: Southern analysis of T_3 transgenic lines derived from a single T_2 plant. A PCR fragment amplified using primers gus F1 and WSMV-R from the pANDA-WSMV CP hairpin plasmid was used as a probe. Lanes are (left to right) DNA size marker, 195A20:2, 110B4:1, and pANDA- WSMV CP HP plasmid.

Chapter Three - Supplemental Data on WSMV CP not presented in Chapter 2

(Cruz *et al*., 2014, *In Vitro Cellular & Developmental Biology - Plant* 50 (6): 665-672)

Materials and Methods

PCR analysis of T_6 generation

Three leaf tips, 2.54 cm in length were collected from newly emerged leaves for DNA isolation. Leaf tissue was placed in 96-1.1 mL collection tubes (USA Scientific) with a 3.96 mm steel bead (Abbott Ball Company, West Hartford, CT). Samples were flash frozen in liquid nitrogen and stored at -80°C. Genomic DNA was isolated using BioSprint 96 DNA Plant Kits (Cat. No. 941558) following the manufacturer's instructions (Qiagen, Valencia, CA). Final samples were resuspended in ddH_2O and stored in a -20°C.

PCR analyses were performed to visualize the presence of the WSMV CP transgene. Each PCR reaction contained 10X PCR Buffer (Sigma Life Sciences, St. Louis, MO), 25 mM $MgCl_2$ solution (Sigma Life Sciences, St. Louis, MO), 0.8 mM dNTPs, 10 pmol of each primer (Table 1), 1.25 U Taq DNA polymerase (Sigma Life Sciences, St. Louis, MO), and 200 ng of high-quality template DNA. Amplification conditions were 5 min at 92°C; 30 cycles of 30 s at 92°C, 40 s at 60°C, and 60 s at 72°C; and a final extension at 72°C for 10 min. Products were run on 1% agarose gel containing 0.033ng/mL ethidium bromide in a electrophoresis box with 1X TAE (40mM Tris, 20mM acetic acid, and 1mM EDTA, pH 8) at 125 V for 20 minutes. Gels were visualized with a UV light box and photographed with a digital camera and Kodak 1D image analysis software.

Transgene expression was determined by extracting total RNA and deriving single-stranded complementary DNA (cDNA) by reverse transcription (catalog no. A3500, Promega). cDNA was used as a template for two PCR reactions. Expression of the *gus* linker was determined by using *gus* sense and antisense primers (Table 3.1) to amplify a 636-bp fragment. A second reaction determined genomic DNA presence in the cDNA using α-tubulin primers (Li *et al*., 2005).

Genetic Crosses

Crosses were made between transgenic T_4 wheat lines 195A and 110B (Cruz *et al*., 2014) and the winter wheat cultivar 'Overley,' released by Kansas State University in 2003 and two elite lines, KS980512 and KS031009, from the Kansas State University wheat breeding program (Fritz, unreleased). 'Overley' is considered very susceptible to WSMV. In the initial cross, the transgenic lines served as the female parent, while 'Overley' and the KS lines served as the male parent. Reciprocal crosses were also made. Resulting crosses were tested via PCR and RT-PCR. These crosses then underwent an additional backcross to their respective recurrent parent.

Bioassays

Plants were inoculated using the finger-rub technique by lightly dusting carborundum onto the second leaf and applying 40 µl of infected plant sap (100 mg of desiccated leaf tissue in 100 µl 0.02 M sodium phosphate buffer, pH 7.4) to the second or third leaf of 2-wk-old seedlings. The leaf was then pinched between the thumb and forefinger and the plant sap was pulled down the length of the leaf ten times. In order to ensure infection, plants were inoculated again 14 d later.

Tissue samples were taken 14 d post second inoculation from the youngest leaf of each plant. One inch of tissue was collected from mid-leaf and placed into a 2 ml screw cap tube

(LabSource, Romeoville, IL). Wheat tissue was evaluated for the presence of WSMV using double antibody sandwich (DAS) ELISA assay. A ceramic bead (MP Biomedical, Solon, OH) was placed in the tube, along with 300 μl of general extraction buffer (Agdia, Elkhart, IN). An MP Fastprep 24 machine (MP Biomedical) was used to grind the leaf tissue at 4 m/s for 20 s. Tubes were spun in a microfuge at 13,000 x *g* for 20 seconds. An additional 700 μl of general extraction buffer was added to the tube. The tube was then vortexed for 15 s and microfuged for 30 s at 13,000 x *g*. Two hundred microliters of supernatant was applied to WSMV Pathoscreen ELISA plates (Cat no. PSA47001) and the manufacturers protocol was followed. Plates were read using a spectrophotometer (Biotek, Highland Park, Winooski, VT) at ABS_{405}. Resistance was determined by comparing ELISA values of each variety to those of both the susceptible and resistant controls. Statistical analysis was performed using SAS version 9.2 (SAS Institute, Cary, NC) and JMP®, Version 11.2 (SAS Institute Inc).

Inoculated and mock-inoculated non-transgenic wheat, as well as transgenic wheat lacking the WSMV CP construct, were used as controls in the bioassays. Any samples with an adjusted value of 2.0 or above were considered positive for the virus. The crosses were grown in greenhouse conditions, 3 seeds each and challenged with the virus. Additional seed from the F_1 cross was then used for an additional cross with the recurrent parent.

Temperature Bioassays

Three replicates of 5 plants of each from T_5 lines originating from the two original events were grown at 18°C, 20°C and 23°C. Plants were inoculated, sampled and analyzed with ELISA as described above.

Results

Analysis was performed as in previous generations, but also included replicated temperature trials. The T_6 plants used originated from two original events, 195A and 110B, and were directly descended from pooled top performing plants found the in the T_5 generation. T_6 transgenic plants were used in 3 replicates at 18°C, 20°C, and 23°C, using 5 seeds each. The varieties 'Tomahawk' (Agripro, 1990) and 'Bobwhite,'(CIMMYT, 1984) and 'Karl92' (Kansas State University, 1992) served as susceptible controls, while the variety 'RonL' (Kansas Agricultural Experiment Station, 2006) served as a resistant control due the presence of *Wsm2*. Susceptible control plants had a 100% disease incidence in every replicate at each temperature. Resistant control, RonL showed evidence of breaking down at 23°C, as expected (Table 3.2). 110B8:9-7.1.1 had two incidences of non-resistant plants. However, of the twenty lines tested at the three temperatures over three replicates, the remainder of the lines showed 100% resistance, despite the temperature.

In total 20 crosses were made from lines. The F_1 seed was recovered from all crosses. These crosses were then grown, underwent PCR confirmation for transgene presence, RT-PCR to confirm transgene expression and challenged with WSMV. The seeds were grown and resulting plants were challenged with the virus. All F_1 crosses were considered resistant (Figure 1). Positive lines were backcrossed to the recurrent parent a second time.

Lines derived from 26 T_5 families derived from event 110B and 22 T_5 families derived from event 195A of the WSMV CP lines were grown under greenhouse conditions or in growth chambers. In total, 201 individual plants were grown. One hundred-eighty eight plants expressed both sides of the WSMV CP hairpin sequences as detected by RT-PCR. Twenty-two plants from 195A:17 contained and expressed the hairpin sequences while two did not express the transgene. Fifty-five plants originating from 195A:20 contained and expressed the hairpin

and three did not express the transgene. Of the 45 plants originating from 110B:4, 43 contained and expressed the hairpin. Sixty-seven plants from family 110B:8 contained and expressed the hairpin, while five did not express the transgene, and one plant died in the pot. Visual inspections show high levels of resistance. ELISA was performed on inoculated samples (Figure 2).

Discussion

Limited genetic resources for resistance to WSMV exist currently. Therefore, a transgenic approach was evaluated for resistance. A hairpin construct was introduced into wheat to target the WSMV CP (Cruz *et al*., 2014). These same lines were continued through the sixth generation. The results showed that T_6 pooled lines are nearly homozygous. These plants also showed significant resistance to the virus. Several possibilities exist to explain the lack of expression in the 12 plants found in the T_6 population. First, particle bombardment can often introduce increased numbers of transgenes, including high-copy numbers of promoters. As evidenced in the original paper, Figure 6, (Cruz et al., 2014) an extreme banding pattern makes it difficult to determine exact copy number found in these lines. It is possible that there are high copy numbers, which continue to lead to events of transgene silencing and DNA methylation (Matzke *et al*., 2000). Furthermore, in experiments, wheat plants that expressed the full-length CP were found to have lost expression by the T_3 generation. This loss of expression was presumed to be attributed to the number of transgene copies generated from bombardment (Li *et al*., 2005). Also, it has been suggested that inverted repeats in transcribed regions are high risk for transgene silencing (Mette *et al*., 1999). This could also be a possible target of genome defense (Srivastava *et al*., 1996). All of these factors can all play a role in transgene silencing

(Hammond *et al*., 2000; Hammond *et al.*, 2001 and Matzke *et al*., 2000). Physical losses of transgenes also occur. Srivastava *et al*., (1996) reported a wheat line that physically lost the transgene in the T_3 generation; however, theT_6 lines of the current study all contained the transgene. While gene silencing is occurring, after stringent selection, T_6 families appear to be completely resistant. Selection at each generation may eliminate lines with predisposition for non-RNAi silencing, so that by the T_6 generation, lines can be considered stable.

To date, there are only three resistance genes effective against WSMV: *Wsm1* and *Wsm3*, both derived from *Thinopyum intermedium*, and *Wsm2*, derived from an unknown source (Haley *et al*. 2006; Seifers *et al*., 2006). The commercially available cultivar 'Mace' contains *Wsm1* (Graybosch *et al*., 2009). *Wsm2* can be found in 'RonL' and 'Snowmass.' Both of these genes contain temperature sensitive resistance to WSMV. *Wsm3* is not currently deployed in any commercially available cultivars, but the gene does confer resistance to the virus at 24°C (Lui *et al*., 2011). This work has confirmed that the transgenic hairpin targeting the CP is in fact effective at temperatures up to 23°C. Field evaluations need to be undertaken, as often transgenes in wheat are not effective under field conditions (Sharp *et al*., 2002). However, the cultivar 'Bobwhite' does not fair well under field conditions, necessitating a cross into an adapted variety. Results indicate that plants generated by conventional crossing methods resulting in the addition of the WSMV CP hairpin into the adapted variety 'Overley' and two KS elite lines were successful, providing preliminary evidence of the applications for field use.

Figures and Tables

Primer name	Sequence (5’-3’)	Product size	Description
WSMV-R	TGCGTGTTCTCCCTCACATCATCT		Used with gus specific primer to determine presence of arms
gus F1	CACGTAAGTCCGCATCTTCA	216 bp + GOI	Used with the CP specific primers to determine presence of GOI
gus R2	GTATCAGTGTGCATGGCTGG	154 bp + GOI	
gus Sense	CATGAAGATGCGGACTTCCG	636 bp	RT-PCR primers to establish *gus* linker expression
gus Antisense	ATCCACGCCGTATTCGG		
Tub-F	ATCTGTGCCTTGACCGTATCAGG	409 bp cDNA 500 bp gDNA	RT-PCR primers internal control
Tub-R	GACATCAACATTCAGAGCACCATC		

Table 3.1: Primer sequence and descriptions of sequences used in these studies.

WSMV Temperature Trials									
Lines	Rep 1			Rep 2			Rep 3		
	18C	20C	23C	18C	20C	23C	18C	20C	23C
195A17:2-4.2.1	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
195A17:2-11.1.1	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
19A17:2-11.2.1	0/5	0/4	0/5	0/5	0/5	0/5	0/5	0/5	0/5
195A17:2-11.2.1	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
195A18:2-11.3.1	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
195A20:2-4.1.1	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
195A20:2-4.2.1	0/5	0/5	0/5	0/5	0/5	0/5	0/3	0/5	0/5
195A20:2-5.4.1	0/5	0/3	0/5	0/5	0/5	0/5	0/5	0/5	0/5
195A20:2-5.4.2	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/4	0/5
195A20:2-201.1	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
110B4:1-8.1.1	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
110B4:1-10.1.1	0/5	0/5	0/6	0/5	0/5	0/5	0/5	0/5	0/4
110B4:1-13.3.2	0/4	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
110B4:1-16.1.4	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
110B8:6-6.4.1	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/4	0/5
110B8:9-4.1.4	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
110B8:9-4.4.1	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
110B8:9-7.1.1	0/5	0/5	1/5	0/5	1/5	0/3	0/5	0/5	0/5
110B8:9-13.2.1	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
110B8:9-14.1.1	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Tomahawk	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5
Karl92	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5
Bobwhite	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5
RonL	0/5	0/5	3/5	0/5	0/5	1/5	0/5	0/5	2/5

Table 3.2: Temperature bioassays of WSMV CP plants derived from single seed selection (Cruz *et al*., 2014) grown in growth chambers at 18°C, 20°C and 23°C. RonL, bearing *Wsm2*, serves as a resistant control for WSMV. 'Tomahawk' and 'Karl92', and 'Bobwhite' serve as susceptible controls. A non-GOI transgenic 'Bobwhite' serves as a susceptible construct control.

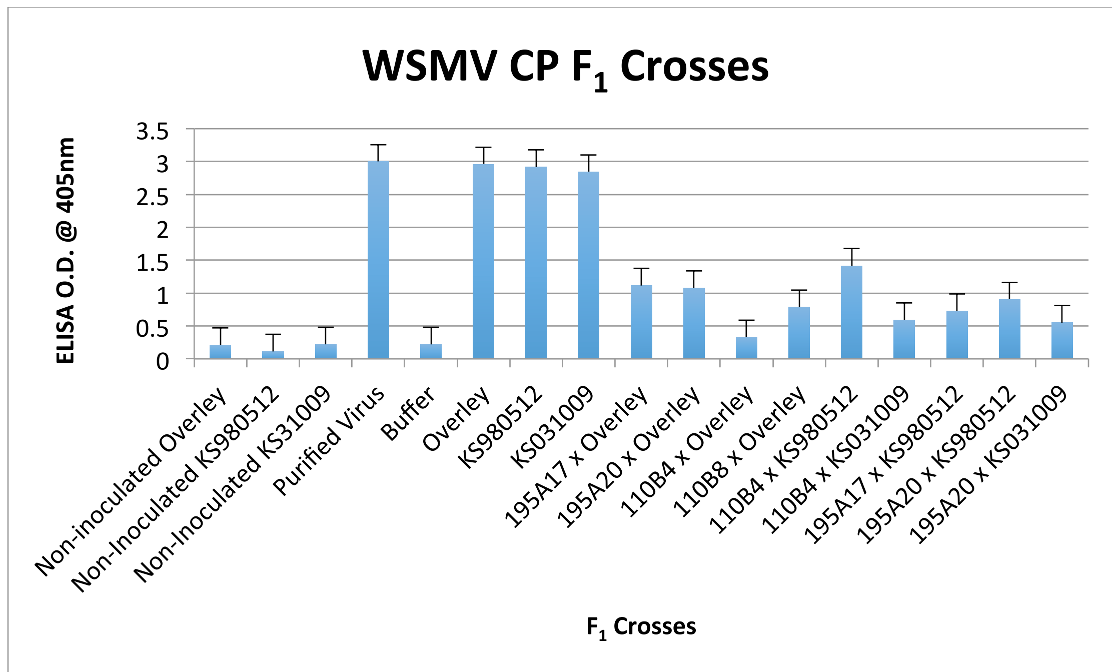


Figure 3.1: F_1 crosses between the transgenic lines and adapted cultivars. Transgenic lines originating from lines 195A17, 195A20, 110B4, and 110B8 serving as the female, and 'Overley,' KS980512, and KS031009 challenged with WSMV represented as ELISA ratings at O.D. @405 nm

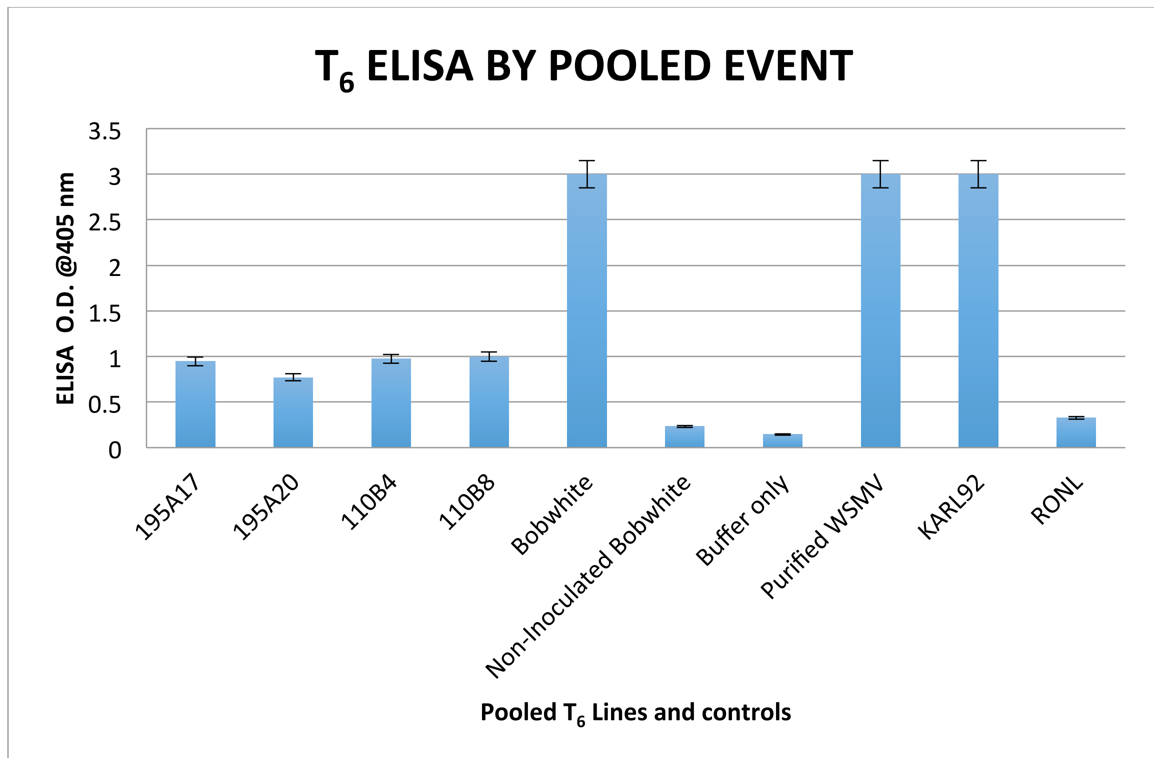


Figure 3.2: ELISA of T_6 generation plants averaged among lineages 195A17, 195A20, 110B4, and 110B8. Non-Inoculated 'Bobwhite' and Buffer only serve as non-infected controls. 'Bobwhite' and 'Karl 92' serve as susceptible controls. RonL, carrying *Wsm*2, serves as a resistant control to WSMV.

Chapter Four - RNAi mediated stable resistance to *Triticum mosaic virus*

Abstract

Triticum mosaic virus (TriMV), discovered in 2006, affects wheat production systems in the Great Plains of the United States. There are few resistant commercial varieties available, requiring disease management strategies focusing on various cultural practices, but these are not fully effective. As an alternative strategy, the use of RNA interference (RNAi) to generate resistance to TriMV was evaluated. An RNAi expression vector was created from the sequence of the coat protein of TriMV. Immature embryos of the wheat cultivar 'Bobwhite' were co-transformed by biolistic particle delivery system with the RNAi expression vector and pAHC20, which contains the *bar* gene for glufosinate selection. After tissue culture and plant recovery, putative transformed plants were analyzed through PCR for the presence of the appropriate RNAi TriMV CP gene. Transgenic T_1 seeds were collected and each line was tested for transgene expression via RT-PCR. To determine viral resistance, T_1 progeny were mechanically inoculated with TriMV. Viral presence was established by DAS-ELISA. In the T_1 generation, resistance was seen in up to 80% of the plants evaluated for the TriMV CP construct. These plants have undergone single plant selection up to the T_6 generation and continue to show high level of resistance when challenged with the virus. Crosses have been made with the virus susceptible winter wheat, 'Overley.' Real-time PCR results show a decrease in viral titer up to 20-fold in the T_6 transgenic lines, the F_1 crosses, and the BC_1F_1 compared to control plants, providing evidence that this RNAi silencing construct can provide stable resistance to TriMV and has great potential benefits to both breeders and producers.

Introduction

Triticum mosaic virus (TriMV), a recent discovery in 2006 (Seifers *et al.,* 2008), is part of the wheat mosaic complex (WMC) affecting much of the Great Plains of the US (Burrows and Stack, 2009). TriMV is the type member of the *Poacevirus,* in the family *Potyviridae* (Tatineni *et al.,* 2009). TriMV is vectored by the wheat curl mite (WCM), *Aceria tosichella* Keifer, along with *Wheat streak mosaic virus* (WSMV) and Wheat mosaic virus (WMoV), but can be mechanically transmitted. TriMV symptoms include green and yellow mosaic patterns on the leaves, yellowing, leaf curling, and stunting (Price *et al*., 2010). Co-infection of TriMV with WSMV can result in a more severe infection, correlating with higher levels of viral titer to both viruses (Tatineni *et al*., 2010). Total crop losses due to TriMV alone remain uncertain, but losses due to the wheat mosaic complex average 5% throughout the Great Plains region, but can approach 100% in heavily infected fields (Christian *et al*., 1993). TriMV infection can cause both significant grain yield reduction and volume weight reductions (Seifers *et al*., 2011). To date, there has only been one resistance gene reported to be effective against TriMV. This relatively new source of resistance was derived from a wheat-*Thinopyrum intermedium* (Host) Barkworth & D.R. Dewey ditelosomic addition line. This Robertsonian translocation was obtained by the 7A#3L arm translocated to the short arm of wheat chromosome 7B. This resulted in the T7BS-7S#3L. The T7BS·7S#3L homozygous stock is capable of conferring resistance to TriMV at 18°C, but is not effective above 24°C. Based on chromosome position and effective resistance to WSMV at 24°C where both *Wsm1* and *Wsm2* are ineffective, the new gene was designated as *Wsm3*. The limited genetic sources of resistance require the primary method of disease control to fall upon the use of cultural practices. These practices include delayed planting dates and eliminating volunteer wheat (Conner *et al*., 1991; Harvey *et al*., 1994). Chemical control of curl mites has been proven ineffective (Kantack and Knutson, 1958).

Plants have an internal system to regulate gene expression called post-transcriptional gene silencing (PTGS). PTGS is also regarded as an ancient method of viral defense. In this system, plants recognize double-stranded mRNA as foreign, thereby targeting the mRNA for degradation (Poogin *et al*., 2001). Specific use of DNA and RNA viruses, as well as transgenes that form hairpins can all induce RNAi silencing to occur (Smith *et al*. 2000; Sivamani *et al*. 2000; Bucher *et al*. 2006; Fahim *et al*., 2010; Cruz *et al*., 2014). Great improvements in transgenic virus resistance have been made utilizing this system (Waterhouse *et al*., 1998). Successful use of transgenes that form hairpins, or dsRNA have been made based on virus sequences (Kalantidis *et al*., 2002; Di Nicola-Negri *et al*., 2005; Tougou *et al*., 2006; Fahim *et al*. 2010; Cruz *et al*., 2014). In this scheme, induction by the dsRNA is first required, which leads to the processing of the dsRNAs into 21-25 nucleotide small interfering RNA (siRNA) by DICER-like enzymes. Finally, the siRNAs are incorporated in the RNA-induced silencing complex (RISC), which will ultimately lead to the degradation of target mRNAs homologous to any previously incorporated siRNA (Hammond *et al*., 2001; Campbell and Choy, 2005; Ruiz-Ferrer and Voinnet Olivier 2007). This method has been successfully used in wheat against WSMV, another potyvirus. Hairpins derived from the viral gene *NIa* (Fahim *et al*., 2010) and the coat protein (CP) gene (Cruz *et al*., 2014) both resulted in wheat resistant to WSMV. The objective of this work was to utilize an RNAi hairpin containing a portion of the CP gene sequence of TriMV to induce viral resistance in transgenic wheat, as well as to determine the stability of this transgene over multiple generations.

Materials and Methods TriMV CP

Cloning and plasmid construction

Winter wheat cultivar 'RonL,' (Kansas Agricultural Experiment Station, 2006) was used to maintain TriMV. Samples of TriMV-infected 'RonL,' isolate U06-123 (Seifers *et al*, 2008; Fellers *et al*., 2009) were obtained from Dr. Dallas Seifers (Kansas State University, Hays, KS). One hundred mg of symptomatic leaf tissue was collected for mRNA extraction. Samples were flash frozen in liquid nitrogen, then ground. One mL of TRIZOL® (Life Technologies, Carlsbad, CA) was added and underwent homogenization. The samples were incubated at room temperature for five minutes. Two hundred microliters (µl) of chloroform was added to each sample. Samples were then vortexed for 30 seconds followed by centrifugation at 12,000 x *g* in an Eppendorf 5415 C centrifuge at 4°C for five minutes. Five hundred µl of 100% isopropyl alcohol was added in order to precipitate total RNA, which was then washed with 500 µl of 70% EtOH and resuspended in 40 microliters of diethyl pyrocarbonate (DEPC)-treated water. A NanoDrop spectrophotometer (NanoDrop Technologies, Rockland, DE) was used to measure RNA concentration. Reverse transcription (RT)-PCR was performed. One microgram of total RNA was denatured at 65°C for 15 minutes. The sample was then placed on ice for five minutes. Five mM $MgCl_2$, 1X reverse transcription buffer (10mM Tris-HCL pH 9.0, 50mM KCl, 0.1% Triton® X-100), 1mM of each dNTP, 1U/µl recombinant RNasin ribonuclease inhibitor, 1 U AMV reverse transcriptase and 0.5 µg random primers/µg RNA (Promega, Madison WI) was added to the sample. Room temperature incubation was performed for ten minutes followed by an incubation period of one hour at 42°C. Samples were then heated to 95°C for 5 minutes and transferred to ice.

The sequence of the CP of TriMV was used design primers using Integrated DNA Technologies, Primer Quest and OligoAnalyzer 3.0 program (www.idtdna.com). Primer sequences for TriMV CP gene segment amplification were selected using forward (TriMV F) and reverse (TriMV R) primers (Table 4.1). For directional cloning of the resulting PCR fragment into the entry vector pENTR-D/TOPO, (Life Technologies), CACC was added to the 5′ end of both forward primers. The CP fragments were independently cloned into the pANDA-mini vector (Miki and Shimamoto, 2004). This was facilitated by homologous recombination using LR Clonase (Invitrogen, Carlsbad, CA). JM109 competent *Escherichia coli* (*E. coli*) cells (Promega) were used for plasmid transformation. An E.Z.N.A. Plasmid Mini Kit (Omega Bio-Tek, Norcross, GA) was used for plasmid purification. Sanger sequencing was used to confirm sequence presence and orientation (Kansas State University DNA Sequencing and Genotyping Facility).

Plant transformation and culture

The spring wheat cultivar 'Bobwhite' (CIMMYT, 1984) was used for tissue culture. Immature seeds (10–14 d post anthesis) were harvested from plants grown under greenhouse conditions. Seeds were first surface sterilized in a solution consisting of 20% v/v sodium hypochlorite (6%) and 0.04% v/v Tween-20 solution for twenty minutes. Embryos were excised from the seeds and placed on callus induction medium CM4 (Zhou *et al*., 1995) in the dark at room temperature. One week later, the scutellum was inspected for organized callus tissue presence. Organized callus, placed on fresh medium, and dried for one hour with petri lid removed to plasmolyze the cells was used for particle gun bombardment. Particle gun bombardment was performed as described by Anand *et al*. (2003a, b). Embryos were co-bombarded with both the RNAi expression vector containing the CP fragment and the vector

pAHC20 containing the *bar* gene (Christenson and Quail, 1996), which confers ammonium glufosinate resistance. All tissue culture methods were as described in Anand *et al*. (2003a). Five bombardments were performed using 180 embryos on each occasion.

Molecular Characterization

Putative transgenic plants were transferred to soil and allowed an acclimatization period of one week. A single leaf from putative T_0 plants were brushed a 0.2% v/v Liberty™ (glufosinate) solution (AgroEvo USA, Wilmington, DE). Herbicide was applied onto one-third of the surface area of a single leaf using a small cotton-tipped applicator. Three days later, plants were inspected. Those plants with an absence of necrosis underwent DNA isolation. An E.Z.N.A. Plant DNA Kit (Omega Bio-tek) was used to isolate genomic DNA (gDNA) from 10 mg of leaf tissue. Forty µl gDNA was eluted water pre-warmed to 65°C. PCR detection of *bar* and CP constructs was performed using 200 ng of high quality gDNA per reaction. Subsequent generations were analyzed with a high-throughput method. Leaf tissue was collected (3–6 cm^2) from each plant and placed in a 96-1.1mL collection racks (USA Scientific). One 3.96 mm steel bead (Abbott Ball Company) was added to each tube. Samples were flash-frozen in liquid nitrogen and stored at -80°C. gDNA was isolated using BioSprint 96 DNA Plant Kits (Cat. No. 941558) following the manufacturer's instructions (Qiagen, Valencia, CA). Final samples were resuspended in ddH_2O and stored in a -20°C.

PCR analyses were performed to detect the presence of the *bar* gene from pAHC20 and the TriMV RNAi CP transgene. PCR reactions contained 10X PCR Buffer (Sigma Life Sciences, St. Louis, MO), 25 mM $MgCl_2$ solution (Sigma Life Sciences, St. Louis, MO), 0.8 mM dNTPs, 10 pmol of each primer (Table 4.1), 1.25 U Taq DNA polymerase (Sigma Life Sciences, St. Louis, MO), and 200 ng of high-quality template DNA. Samples were amplified with the

following conditions: were 3 min at 92°C; 34 cycles of 1 min at 92°C, 2 min at 60°C, and 2 min at 72°C; and a final extension at 72°C for 10 min. PCR products were run on 0.8% agarose gel containing 0.033ng/mL ethidium bromide in a electrophoresis box with 1X TAE buffer (50X 242 g TRIS base, 57.1 ml acetic acid, 100 mL 0.5 M EDTA pH 8.5), at 125 V for 20 minutes. Resulting gels were visualized using a UV light box and photographed with a digital camera and Kodak 1D image analysis software.

Total RNA was extracted and single-stranded complementary DNA (cDNA) was made using reverse transcription (catalog no. A3500, Promega) to determine transgene expression. cDNA was used as a template for PCR reactions to detect the expression of the *gus* linker sequence using gus sense and antisense primers (Table 1) to amplify a 636-bp fragment. A second reaction determined the presence and replication of viral RNA by PCR to detect the cylindrical inclusion (CI) gene fragment of TriMV. Genomic DNA presence in the cDNA was determined using α-tubulin primers (Li *et al*. 2005). Data from plants from the same event were pooled and tested using a chi-square test. Southern analysis was performed as in Faris *et al*, (2000).

Bioassays and ELISAs

Wheat plants at the three-leaf stage were used for bioassays. One gram of infected 'RonL' was ground in 20 ml 0.02 M phosphate buffer, pH 7.2. The filtrate was passaged through cheesecloth. The adaxial surface of the second wheat leaf was sprayed with a Devilbiss atomizer sprayer (Devilbiss, Long Grove, IL) containing the filtrate and 0.3 g carborundum powder at 25-30 psi until a "water-soaked" lesion was visible. Following inoculation, plants were placed in a growth chamber with 16-h day/8-h night, 500 $\mu E/m^2/s$ light intensity, at 19°C. Samples of leaf tissue were taken 14–21 d post-inoculation. All following generations were

inoculated using the finger-rub technique. Carborundum was rubbed on the second or third leaf of two-week old seedlings using a cotton-tipped applicator. Then, 40 µl of infected plant sap (100 mg of desiccated leaf tissue in 100 µl 0.02 M sodium phosphate buffer, pH 7.4) of 2-wk-old seedlings was dotted on the leaf and leaf was pinched between the thumb and forefinger. The plant sap and carborundum was pulled down the length of the leaf ten times. Plants were inoculated again 14 days later to ensure infection. An indirect ELISA was performed. Leaf tissue was ground in a 1.5-ml microcentrifuge tube using a micropestle at 1:30 (wt/vol) in 0.05 M carbonate buffer, pH 9.6 (Clark *et al*., 1977). Extracts (200 µl) were placed in wells of ELISA plates (Immulon 1, catalog no. 1424578; Fisher Scientific) for 1 h at 37°C. Following rinsing, the wells were incubated for 1 h at 37°C with the TriMV antibody (Agdia, Elkhart, IN) in dilution buffer (Clark *et al*., 1977). The plates then were rinsed and blocked for one hour in blocking buffer (5% nonfat dry milk, 0.01% antifoam A, and 0.02% sodium azide, in phosphate-buffered saline, pH 7.4) at 37°C. Then, 200 µl of anti-rabbit antibody/alkaline phosphatase conjugate (Agdia, Elkhart, IN) in dilution buffer (1:3,000 vol/vol) was added to each well. The plates were placed at 37°C for 1 h. Following another rinse, 200 µl of p-nitrophenyl phosphate substrate at 0.714 mg/ml, in substrate buffer (Clark *et al*., 1977), was added to each well. The plates then were held at ambient temperature for 30 min. Absorbance was measured at 405 nm using a Biotek EL-800 Absorbance Reader (Biotek, Highland Park, Winooski, VT). Samples from inoculated and mock-inoculated non-transgenic wheat, along with inoculated transgenic samples, and transgenic inoculated wheat without RNAi construct against TriMV were used in the assays. Adjustments were made to the raw ELISA readings in order to standardize across multiple plates using the following formula: (sample – blank)/(negative control – blank). Any samples with an adjusted value of 2.0 or above were considered positive for the virus (Zhang *et*

al., 2011). T_3, T_4 and T_5 plants were measured for height individually. Plants were allowed to reach full maturity. Seed was harvested from each plant and weighed.

Genetic Crosses

Crosses were made between transgenic wheat and the winter wheat cultivar ‘Overley,’ released by Kansas State University in 2003. ‘Overley’ is considered very susceptible to TriMV. In the initial cross the transgenic lines served as the female parent, while ‘Overley’ served as the male parent. Reciprocal crosses were also made. Resulting crosses were tested via PCR and RT-PCR. These crosses then underwent an additional backcross to the recurrent parent.

Resulting crosses were grown in greenhouse conditions, 5 seeds each and challenged with the virus. An experiment was performed in three replicates containing 5 plants of mock-inoculated ‘Bobwhite’, 5 plants of inoculated ‘Bobwhite’, 5 plants originating from 817F4A, 5 plants F1 817F4A x Overley, 5 plants of BC1F1’s, and 5 plants of ‘Overley’ were used in each replicate. Each of these lines was grown under greenhouse conditions and challenged with the virus. Plants were measured for height and seed weight.

Real-time PCR Screening

Real-time RT-PCR was performed on inoculated and mock inoculated ‘Bobwhite’ samples, transgenic samples, crosses, backcrosses, as well as the parental ‘Overley.’ Three experimental replicates were performed. In each experiment, 10 plants of ‘Bobwhite,’ 5 plants originating from the T_3 line 817F4A, 5 plants from the 817F4A x Overley F_1 cross, 5 plants of the BC_1F_1’s, and 5 ‘Overley’ used from the crossing were grown. Five of the ‘Bobwhite’ plants and all of the transgenic lines, crosses, and ‘Overley’ were inoculated with virus, while the remaining 5 plants of ‘Bobwhite’ served as a mock-inoculated control. The plants were all grown under greenhouse conditions and challenged with the virus as described above. At 14 d

post second inoculation, plants were scored for visual symptoms, and sampled for RNA. RNA samples from each line were pooled independently from each replicate. One µg of total RNA was used to complete first strand synthesis with random hexamers, followed by reverse transcription with Superscript II (Invitrogen) according to the manufacturer's recommendations. Primers for qRT-PCR were designed from the gene sequence of *Triticum mosaic virus NIb* (NCBI Reference Sequence: NC_012799.1) and used to assess differences in the levels of viral titer between samples. iQ SYBR Green Supermix for iCycler (BioRad, La Jolla, CA) was used in all reactions. Plants were then analyzed using real-time PCR. Values of relative expression of the viral gene *NIb*, representing reduction in viral titer were calculated using the ΔΔCt method (Schmittgen and Livak, 2008). Three technical replicates for each biological replicate were performed for each reaction. Primer efficiencies were tested prior to experimental use. Primers with efficiencies within the range of 90-110% were considered appropriate for use in experiments. The resulting Cq value for the target was subtracted from the Cq value of the internal reference gene *Actin* (GenBank accession #AB181991) (Table 1).

Temperature Bioassays

Three replicates of 5 plants of each from T_4 lines originating from the two original events were grown at 18°C, 20°C and 23°C. Plants were inoculated at both the two-leaf stage and two weeks later. Fourteen days following the second inoculation, plants were sampled and analyzed as described above.

Results

A 272 bp segment of the CP gene of TriMV was amplified and cloned into pANDA-mini vector. Five independent biolistic transformation experiments were performed using approximately 900 wheat callus explants and 176 putative transgenic plants were regenerated.

These plants were screened using the herbicide glufosinate. Sixteen plants were determined to putatively contain the *bar* gene, and underwent PCR analyses using genomic DNA (Figure 2). Eight plants were found to contain the complete hairpin construct. Seeds of the T_0 plants were independently collected. Twenty seeds of each of the T_0 lines of the T_1 generation were planted; at the two-leaf stage, leaf samples were taken, pooled and used to reconstitute the T_0 generation for PCR and RT-PCR testing performed at a later date to confirm transgene presence and expression.

In the T_1 generation, each of the lines was found to contain the transgene via PCR, and expressing the transgene to varying degrees. Segregation in these lines fit a 3:1 ratio (data not shown), suggesting integration at a single locus. These plants were grown and challenged with TriMV. At 21 d after the second inoculation, samples from the youngest leaves were taken for molecular analysis and symptoms were recorded. However, when challenged with the virus, only lines originating from two events, 201 and 817 had high levels of resistance, while the other six events were varied, but considered moderately to severely susceptible. Each tiller of the T_0 had been harvested separately, and given a letter designation for selection records, while each plant of the T_1 generation and beyond was selfed and harvested separately. Lines 201A, 817A, 817D and 817F had high levels of resistance and were selected for analysis in the T_2 generation. Selected T_2 seeds were planted and resulting plants were given a number designation.

In the T_2 generation, several plants did not exhibit a resistant phenotype. Additionally, some of the plants derived from resistant plants failed to be resistant in the T_2 generation. Only one individual plant originating from event 201, 201A19, kept a high level of resistance. Line 817F showed a consistent resistant phenotype through all progeny grown from the T_2 generation. Viral RNA could be detected in plants with symptoms via ELISA and RT-PCR. The negative-

control non-transgenic plants, transgenic non-CP plants, and control varieties showed 100% virus incidence. Plants were rated on a (1-9) scale, with 9 being extremely susceptible. Single seeds from individual transgenic plants were chosen based on the criteria that they contained and expressed the transgene, had a rating of 4 or below on the phenotypic rating scale, and had ELISA scores comparable to that of buffer only wells. However, the only plant meeting this criteria originating from event 201 produced only 11 seeds.

In the T_3 generation, 5 seeds of plant 201A19 were grown, as well as 495 seeds originating from event 817. In total, 405 plants contained the TriMV CP transgene. Line 817 produced 368 plants that were considered resistant, while 4 plants from line 201 were resistant using the same analysis scheme as in the T_2 generation. Individual plants were given a letter designation in this generation.

Single seed selection was performed to generate a T_4 population consisting of 332 individual plants, 40 originating from event 201, and the remaining plants from event 817. Two hundred and eighty-nine plants contained the transgene, and 265 were considered resistant, including 35 from event 201. All plants in the T_3 and T_4 generation were measured for height and seed weight individually. Analysis revealed that there was no statistical difference in height or per plant seed weight between the GOI positive, expressing transgenic lines and the non-inoculated controls ($p=0.495$). However, a significant difference did exist between the susceptible controls and those transgenic lines no longer containing or expressing the transgene and/or no longer resistant ($p<0.05$) (Figure 4).

T_5 analyses (i.e. PCR, RT-PCR, bioassays) were performed as in previous generations, but also included replicated temperature trials at 18°C, 20°C, and 23°C. T_5 plants used originated from the two original events chosen, 201 and 817, and were directly descended from

top performing plants found the in the T_4 generation, which were then pooled. T_5 transgenic plants from T_4 lines were used in 3 replicates at above temperatures, using approximately 30 seeds each. The varieties 'RonL', 'Tomahawk' and 'Bobwhite' served as susceptible controls. Control plants had a 100% disease incidence in every replicate at each temperature. The best performing lines included 817F4A, 817F4B, 817D6A, and 817H1A (Table 2). Southern analysis of the T_5 lines showed multiple copies of the transgene (Fig. 5).

Several lines in the T_3 generation were chosen for crossing with the winter wheat variety, 'Overley.' The reciprocal cross was also made from the same plant. In total 22 crosses were made from lines 817F4A, 817F4B, 817D10A and 201A19A. The F_1 seed was recovered from all crosses from event 817, but no seed was recovered from event 201. These crosses were then grown, 5 seeds each and challenged with the virus. All F_1 crosses were considered resistant. Additional seed from the F_1 cross was then used for an additional cross with the recurrent parent. These BC_1F_1's were grown, underwent PCR confirmation for transgene presence, RT-PCR to confirm transgene expression and challenged with TriMV. All BC_1F_1's were resistant to the virus. In replicated experiments mock-inoculated 'Bobwhite', inoculated 'Bobwhite', 817F4A, F_1 cross 817F4A x 'Overley', the BC1F1's, and 'Overley' were grown under greenhouse conditions and challenged with the virus. Inoculated 'Bobwhite' and 'Overley' showed 100% disease incidence, received a '9' severity rating, were extremely stunted and produced little to no seed (Figure 6). Each of the transgenic lines, transgenic crosses and mock-inoculated 'Bobwhite' displayed no visible symptoms, the CP was undetectable with ELISA, and no viral RNA was detected via RT-PCR. Plants were then analyzed using real-time PCR (Figure 7).

Resistant plants were selected from the T_5 families and grown for the T_6 and the progeny were again resistant. Presence of the TriMV CP transgene as determined by PCR was evaluated and following viral challenge was evaluated via ELISA (Figure 8). Stable resistance was evident.

Discussion

This study is the first report of RNAi-mediated resistance to TriMV in transgenic wheat and the second report of the stability of this type of transgene functioning over many generations (six); the first being Cruz *et al*., (2014) studying transgenic wheat containing a hairpin derived from the gene sequence of the CP of *Wheat streak mosaic virus*. In this work, it is assumed that the hairpin sequence of the TriMV CP gene is responsible for the resistance. This is hypothesized to be attributed to RNAi induction by the dsRNA. This method has been shown to be effective in wheat using other potyviruses and gene sequences. In WSMV, Cruz *et al*., plant lines were carried into the T_5 and successful resistance was maintained utilizing a hairpin derived from the CP. Fahim *et al*., (2011), also targeting WSMV, was successful in generating wheat that maintained resistance to WSMV using a hairpin derived from the *NIa* gene through the T_2 generation. In particular, the use of hairpin constructs has effectively been shown to increase efficiency of PTGS (Smith *et al*., 2000) in comparison to constructs bearing only sense or antisense. Furthermore, RNAi based gene silencing strategies in wheat for functional gene analysis has been shown to reduce the expression of targeted endogenous genes (Fu *et al*., 2007). The use of hairpin constructs targeting potyviral CP's to generate resistance has also been reported in other species. Guo *et al*., (2015) found resistance to *Sorghum mosaic virus* (SrMV) in sugarcane using a hairpin derived from a portion of the CP of SrMV. Resistance has also been shown in cantaloupe to *Papaya ringspot virus Type W* (Krubphachaya *et al*., 2007), in plum against *Plum pox virus* (Hily *et al*., 2007), in Soybean against *Soybean mosaic virus* (Tougou *et*

al., 2006) and in barley against *Barley yellow dwarf virus* (Wang *et al*., 2000) confirming that this is a viable strategy in a variety plant species against a multitude of viruses. These results agree with previous studies that have shown varying degrees of resistance generated using full-length sequences of the WSMV CP (Li *et al*., 2005) or viral replicase, *NIb* (Sivamani *et al*., 2000) in transgenic wheat.

Based on PCR analysis of transgenic lines at various generations, a number of lines have been identified that suggest independent assortment of the *bar* gene and GOI. The utilization of cotransformation using two different plasmids, (in this study: pANDAmini containing the hairpin construct, and pAHC20 containing the *bar* gene) has been shown to facilitate segregation of transgenes independently of selectable marker genes (Komari *et al*., 1996; Matthews *et al*., 2001, Huang *et al*., 2004, Zhao *et al*., 2007, Fahim *et al*., 2011 and Cruz *et al*., 2014). This can be considered advantageous due to that fact in the T_3 generation and beyond marker-free TriMV resistance plants were found. This further allowed the transmission of the transgene into 'Overley' without the selectable marker.

Non-Mendelian ratios are evident in both events carried through the T_5 generation and fail to be explained by segregation of a single dominant gene. It is possible that gene silencing is occurring; however, after several generations of selection, T_5 families appear to be completely resistant. Generational selection may assist to eliminate lines with predisposal for silencing.

Limited genetic resistance exists for use with TriMV. The commercial cultivar 'Mace' contains *Wsm1* (Graybosch *et al*., 2009), however has been shown to contain a significant yield drag when not challenged with viral infection from WSMV. *Wsm3* is not currently deployed in any commercially available cultivars and is unfortunately, not effective at high temperatures. This work has confirmed that the transgenic hairpin is effective at temperatures up to 23°C.

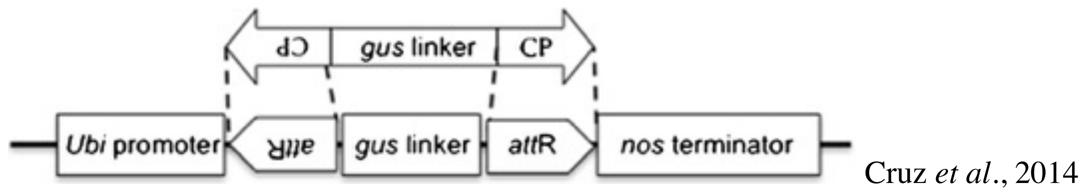
While field evaluations need to be undertaken, as often transgenes in wheat are not effective under field conditions (Sharp *et al*., 2002), this study provides evidence that the transgene is not temperature sensitive. Additionally, results indicate that plants generated by conventional crossing methods resulting in the addition of the TriMV CP hairpin into the adapted variety 'Overley' was successful, providing preliminary evidence of the applications for field use. The behavior of an RNAi hairpin such as this, in a variety of adapted backgrounds remains unclear; however, this research indicates that it is possible in the variety 'Overley.' Further research is required in order to understand how the genome background might affect silencing.

While the use of hairpin constructs for virus resistance is not novel, this is the first report of stable TriMV-resistant wheat produced by this method. In some lines, transgene silencing did occur; however, lines were produced that were stable through the T_6 generation by using multi-generational selection. This work also produced a successful backcross population in the commercial winter wheat cultivar 'Overley' in which resistance was maintained while achieving a winter wheat background. Ultimately, future work is required to determine if this transgene will maintain expression and provide continued resistance under field conditions.

Figures and Tables

Primer name	Sequence (5′′-3′′)	Product size	Description
TriMV-F	CACCGATAGACGATGCGACTGGGCAAAT	272 bp	Amplification of TriMV CP fragment in RNAi vector
TriMV-R	TCTGTTCCTGTGGTGAAAGCTGGT		
gus F1	CACGTAAGTCCGCATCTTCA	216 bp +GOI	Used with the CP specific primers to determine presence of GOI
gusR1	ATCTCTTTGATGTGCTGTGCC	353 bp+GOI	
gusR2	GTATCAGTGTGCATGGCTGG	154 bp +GOI	Used with specific CP primers to determine presence of GOI
gus Sense	CATGAAGATGCGGACTTCCG	636 bp	RT-PCR primers to establish gus linker expression
gus Antisense	ATCCACGCCGTATTCGG		
TriMV2-R	TCTGTTCCTGTGGTGAAAGCTGGT	408 bp	Used in RT PCR to determine virus presence in TriMV transgenic material
TriMV2-F	CGGCAGCAAATGGACTTGGATTGA		
BarABR	CCTGCCTTCATACGCTATTTATTTGC	500 bp	Amplification of *bar* gene
UbiABF	CTTCAGCAGGTGGGTGTAGAGCGTG		
TubF	ATCTGTGCCTTGACCGTATCAGG	409 cDNA	RT- PCR primers internal control used to determine cDNA contamination
TubR	GACATCAACATTCAGAGCACCATC	500bp gDNA	
TriMV qNib-F	GCTGAGTTGAGACCGAAAGAA		Real-time PCR primer to detect TriMV
TriMV qNib-R	GCC TGC CTG TGT AGC ATA AA	135 bp	
Actin qa-F	GTTCTCAGTGGAGGTTCTA		Real-time PCR primer to detect constitive gene
Actin qa-R	CTTTCAGGTGGTGCAATAA	113 bp	

Table 4.1: Primer Sequences and descriptions



Cruz *et al*., 2014

Figure 4.1: Schematic representation of TriMV CP RNAi vector. pANDA-mini (Miki and Shimamoto 2004) vector with attR sites allowing for homologous recombination insertion of inverted TriMV CP sequences flanking a gus linker (Cruz et al., 2014), which then targets the dsRNA sequence for degradation.

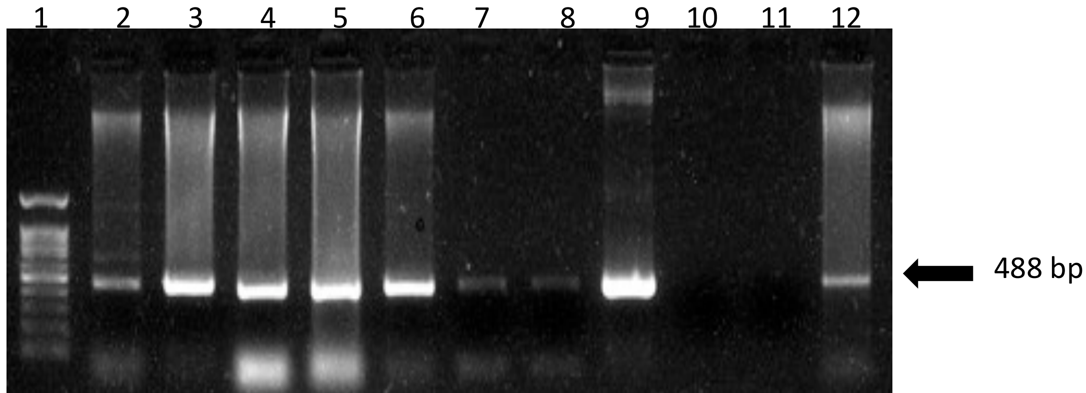


Figure 4.2: PCR analyses on gDNA of T_0 TriMV CP RNAi transgenic wheat reconstituted by T_1 pooling. *gus* forward (*gus* F1) and TriMV-Reverse (TriMV-R) primers were used on genomic DNA. Each plant was resistant to application of glufosinate. Lane 1: Marker, Lane 2: 72 ER, Lane 3: 147, Lane 4: 201, Lane 5: 278, Lane 6: 467, Lane 7: 445 Lane 8: 470, Lane 9: 817 Lane 10: Non-transgenic Bobwhite, Lane 11: H_20 only, Lane 12: Plasmid

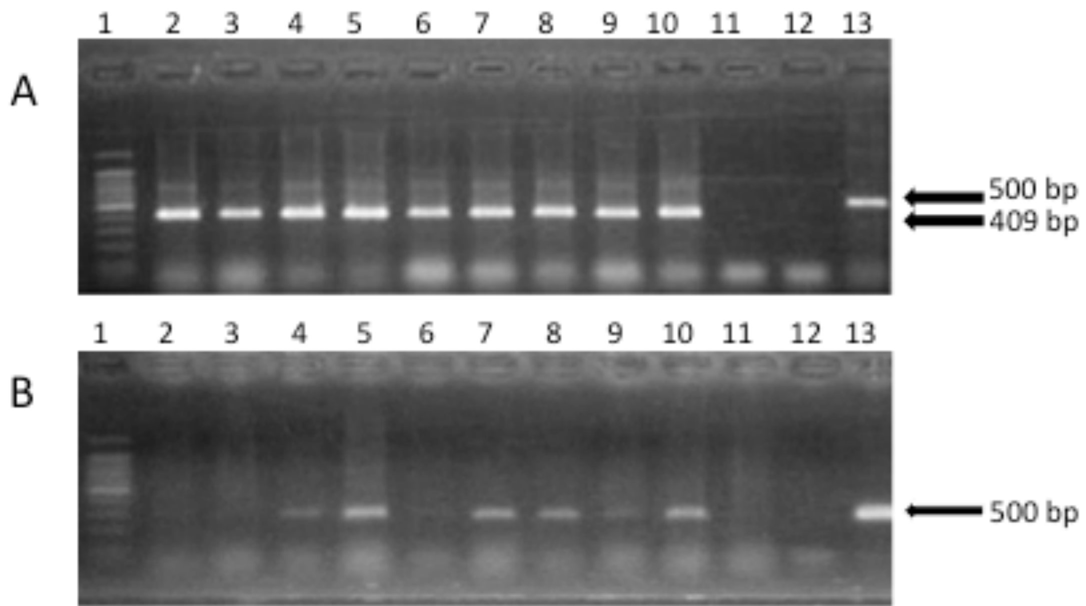
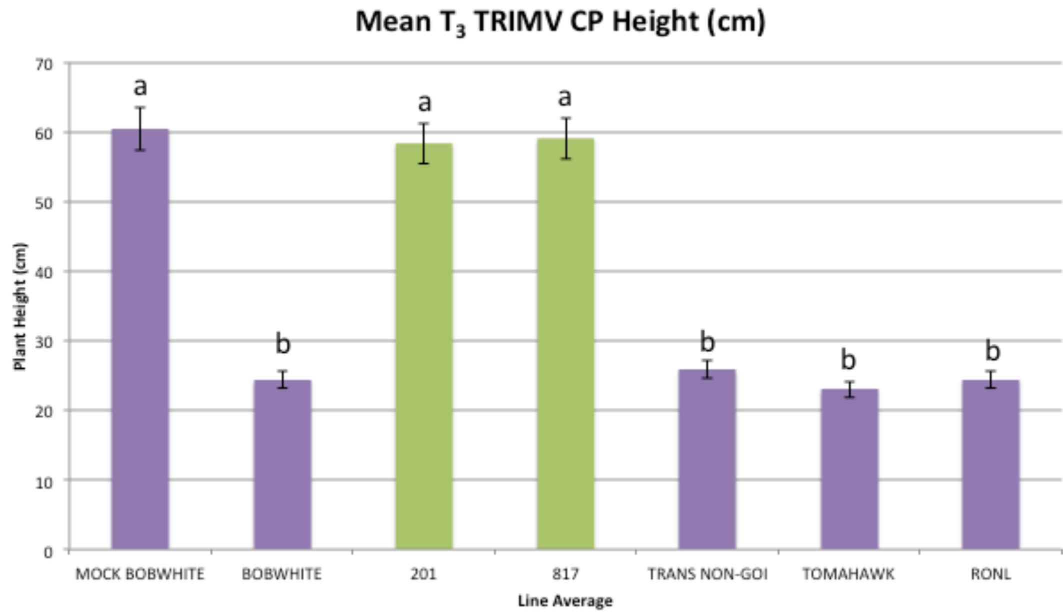


Figure 4.3: RT-PCR analyses on RNA of T_0 TriMV CP RNAi transgenic wheat reconstituted by T_1 pooling. Panel 1: Primers Tubulin Forward (Tub-F) and Tubulin Reverse (Tub-R) were used. Lane 1: Marker, Lane 2: 72 ER, Lane 3: 147, Lane 4: 201, Lane 5: 278, Lane 6: 467, Lane 7: 445 Lane 8: 470, Lane 9: 817 Lane 10: Non-transgenic Bobwhite, Lane 11: H_20 Lane 12: Plasmid Lane 13 gDNA of Non-transgenic Bobwhite; Panel 2: Primers *gus* sense and *gus* antisense were used. Lane 1: Marker, Lane 2: 72 ER, Lane 3: 147, Lane 4: 201, Lane 5: 278, Lane 6: 467, Lane 7: 445 Lane 8: 470, Lane 9: 817 Lane 11: Non-transgenic Bobwhite, Lane 12: H_20 Lane 13: Plasmid



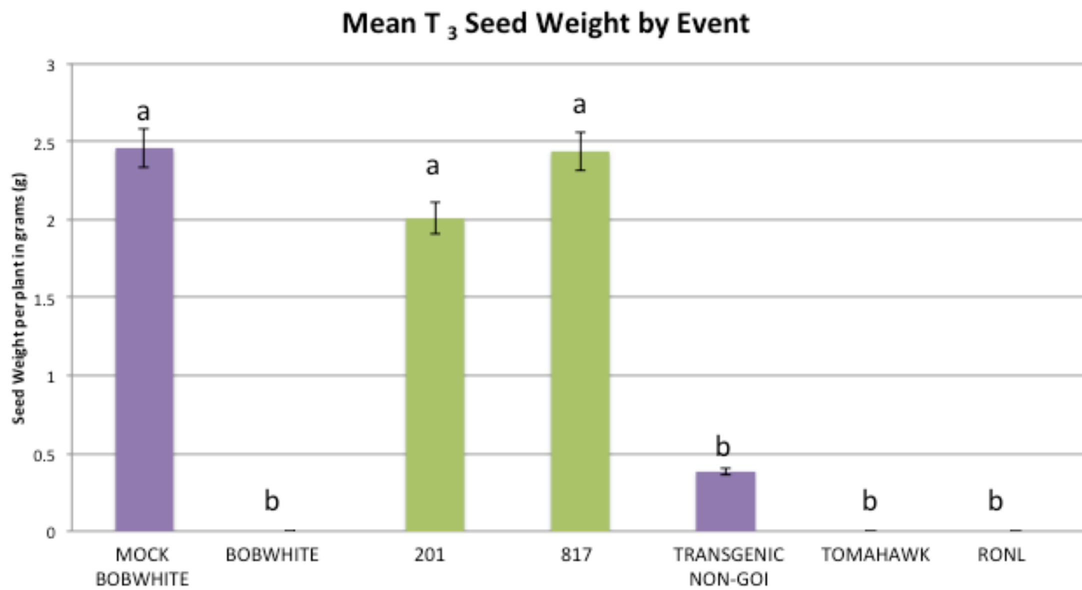


Figure 4.4: T_3 generation analysis of plant height and seed weight. No statistical difference was found between the height or seed weight of transgenic lines and non-inoculated bobwhite, ($p=0.495$), while seed weight and height are statistically ($p<0.05$) different between inoculated controls.

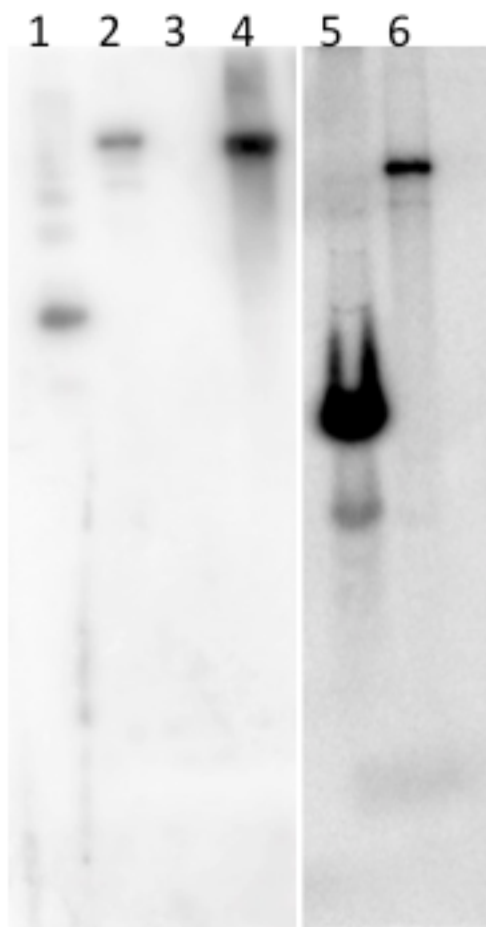
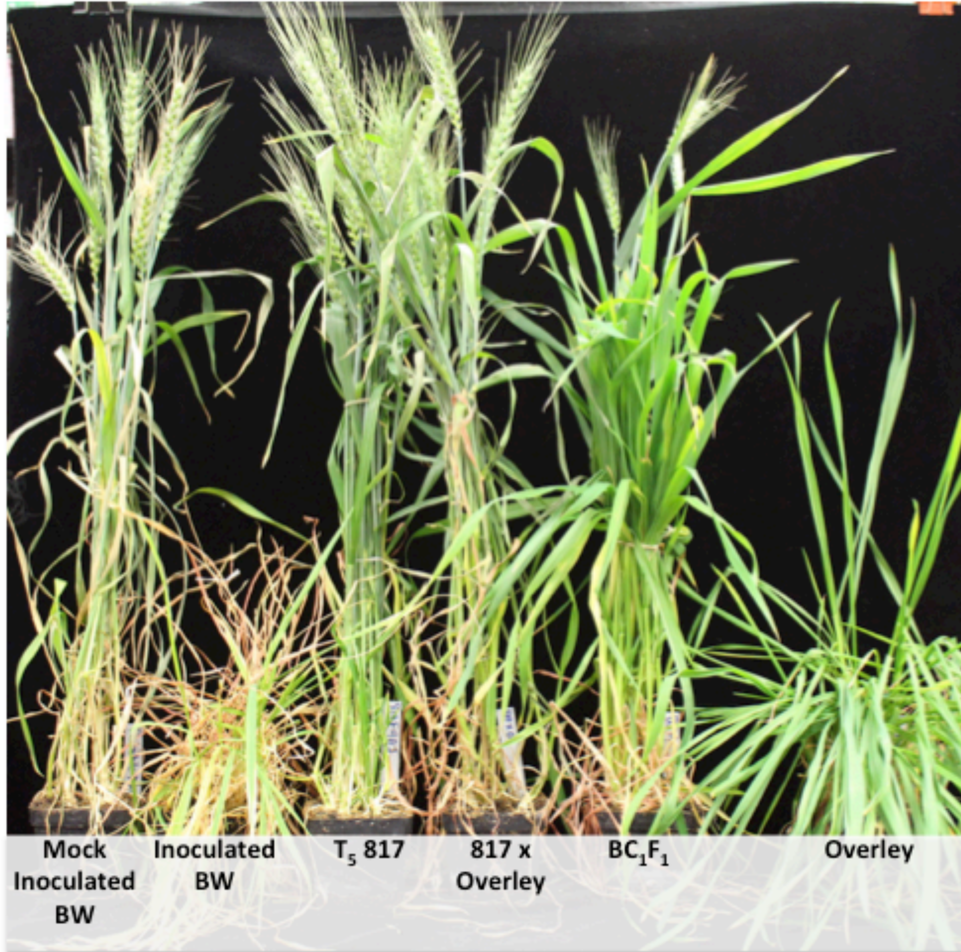


Figure 4.5: Southern Blot analysis: Lane 1: 1 KB $^+$ Ladder, Lane 2: *HindIII* digested Lambda DNA, Lane 3: Non-Transgenic Bobwhite, Lane 4: TriMV CP RNAi plasmid Construct, Lane 5: Event 817, Lane 6: Event 201

A



B

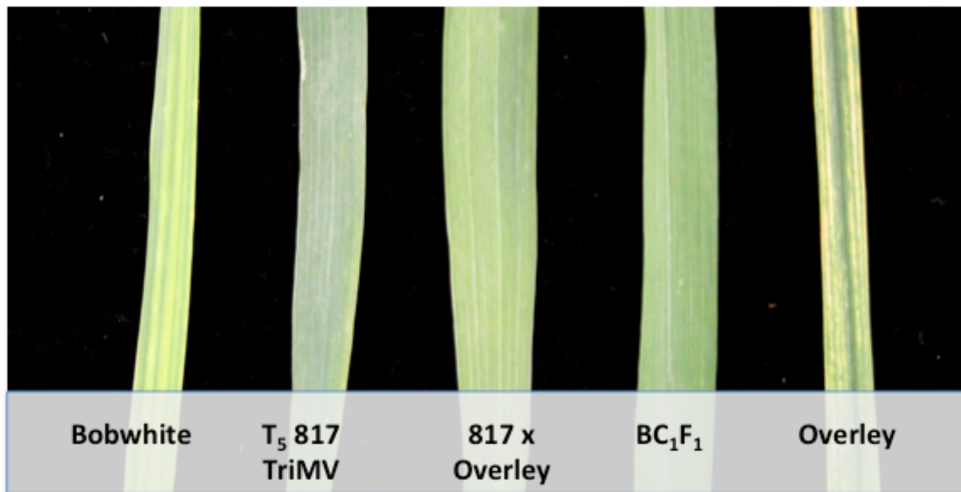


Figure 4.6: T_5 Plants and Crosses Panel A: Mock Inoculated Bobwhite (BW), Inoculated BW, Inoculated Transgenic line 817, Inoculated F_1 Cross: 817 x Overley, Inoculated BC_1F_1, Inoculated Overley Panel B: Bobwhite Inoculated, Transgenic line 817, Inoculated F_1 Cross: 817 x Overley, Inoculated BC_1F_1, Inoculated Overley

TRIMV CP TEMPERATURE TRIALS									
	REP 1			REP 2			REP 3		
LINES	**18°C**	**20°C**	**23°C**	**18°C**	**20°C**	**23°C**	**18°C**	**20°C**	**23°C**
201A19	0/27	2 /30	0/28	1/28	2/30	2/28	4/30	2/26	0/24
817F4A	0/30	0/31	2/ 28	0/30	0/26	0/30	0/30	0/29	0/30
817F4B	0/22	0/15	0/30	0 /24	0/23	0/24	0/30	0/27	0/29
817C5A	0/28	0/28	0/26	0/30	0/30	1/29	2/30	2/28	3/28
817D6A	0/29	1 /30	0/27	2/30	1/29	2/30	0/30	0/29	0/30
817E5F	0/30	0/23	1 /16	0/27	0/29	0/28	1/29	0/30	2/30
817G2D	0/26	0/30	0/27	1/28	1/23	1/24	0/27	2 /26	2/30
817H4A	0/26	1/19	0/29	2/24	1/24	1/18	0/26	0/28	0/26
RONL	30/30	30/30	30/30	25/25	30/30	28/28	24/24	30/30	30/30
TOMAHAWK	28/28	30/30	24/24	28/28	29/29	24/24	26/26	25/25	27/27
BOBWHITE	30/30	30/30	26/26	30/30	30/30	28/28	39/39	30/30	30/30

Table 4.2: Temperature bioassays of T_5 TriMV CP plants derived from single seed selection grown in growth chambers at 18°C, 20°C and 23°C. ‘RonL,’ ‘Tomahawk,’ and ‘Bobwhite’ serve as susceptible controls. Columns represent the total number of ELISA positive plants/ total plants.

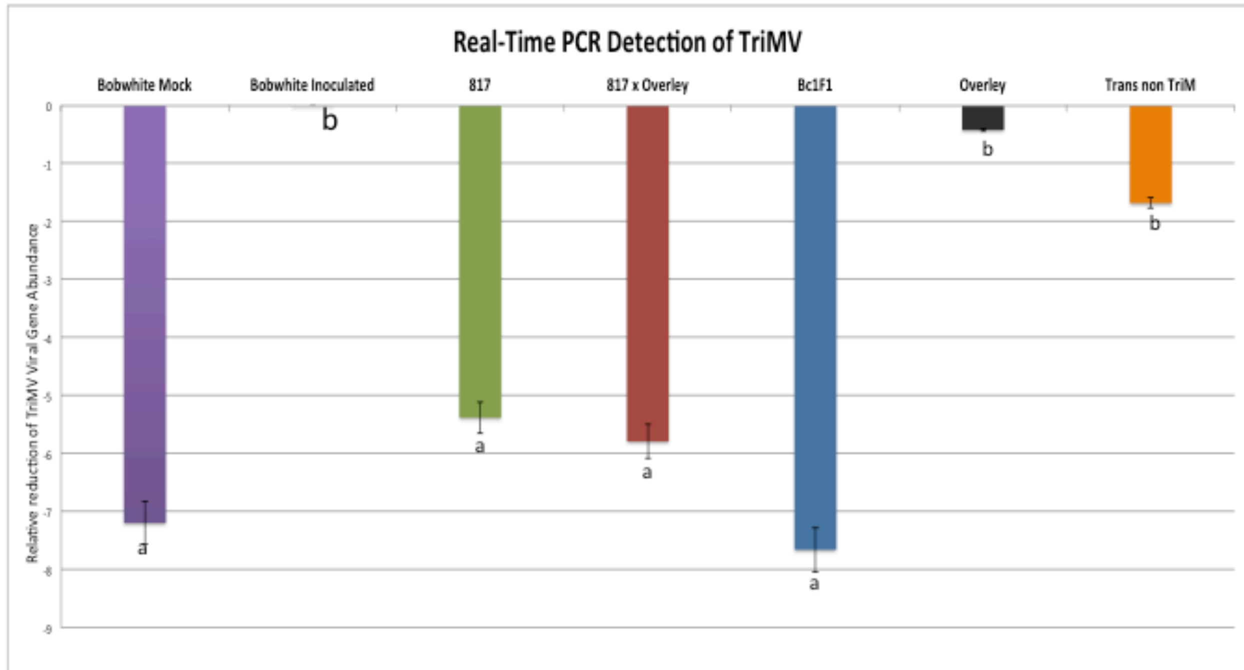


Figure 4.7: Relative reduction of TriMV viral gene *NIb* abundance using real-time PCR in comparison to inoculated 'Bobwhite.' Five plants of each line were grown, and inoculated with TriMV at 14 days, and 28 days. Forty-two days after the initial inoculation plants were sampled for analysis. Analysis was performed using the ΔΔCT method. Three biological replicates were performed with three technical replicates in each. This was repeated for a total of three experimental replicates. The graph displays the average values among the three experimental replicates. Mock-inoculated 'Bobwhite' (Bobwhite Mock), Line 817, the F_1 cross, 817 x Overley, and the BC_1F_1 were considered to be nearly undetectable ($p<0.05$), in comparison to inoculated Bobwhite, as well as virus-susceptible parent, 'Overley', and a transgenic line without the GOI in a 'Bobwhite' background.

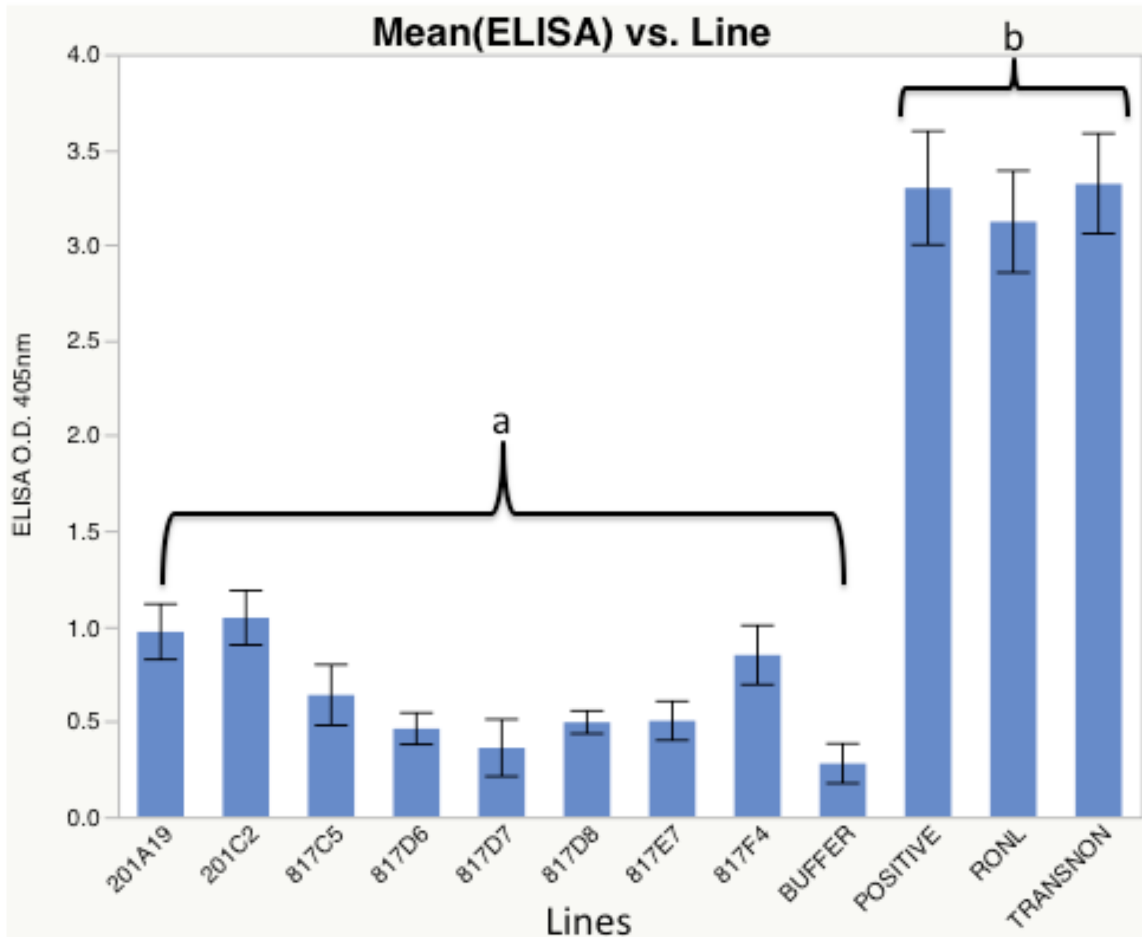


Figure 4.8: Indirect ELISA detection of the *Triticum mosaic virus* coat protein antigen in in T_6 transgenic lines. Plants were grown for the T_6 generation and inoculated with TriMV at 14 and 28 days. Forty-two days after the initial inoculation, plants were sampled for ELISA. Purified virus (POSITIVE), 'RonL' and transgenic 'Bobwhite' lacking the TriMV CP construct (TRANSNON) were used as controls. ELISA values were averaged by T_1 family and Dunnett's Adjustment was used to compare each of the families to the buffer control ($p<0.05$).

Chapter Five - RNAi mediated silencing of endogenous wheat genes *eIF(iso)4E-2* and *eIF4G* induce resistance to multiple RNA viruses in transgenic wheat

Abstract

The eukaryotic initiation factor (eIF) complex plays important roles in recruitment of mRNA to the ribosome in wheat (*Triticum aestivum* L.) and all other plants. Several plant-infecting viruses, which lack sufficient proteins to compete their lifecycle, have been shown to interact with this complex in order to facilitate replication of their genomes. The use of RNAi to silence eIF(iso)4E-2 and eIF4G to interrupt this process in order induce resistance to these multiple wheat viruses was evaluated. RNAi expression vectors were independently created from the sequences of the wheat genes eIF(iso)4E-2 and eIF4G. Immature embryos of the wheat cultivar 'Bobwhite' were independently co-transformed using each of the RNAi expression vectors and pAHC20, which contains the *bar* gene for glufosinate selection using particle bombardment. All progeny have undergone PCR and RT-PCR analysis. Viral resistance was determined in the progeny by mechanical inoculation with the viruses *Wheat streak mosaic virus* (WSMV) and *Triticum mosaic virus* (TriMV). A consistent stable resistance response was demonstrated in three transgenic lines of eIF(iso)4E-2 construct and four transgenic lines of eIF4G, each derived by single seed descent. T_6 progeny were co-infected with WSMV and TriMV and continue to be resistant. Traditional crosses have been performed with the winter wheat 'Karl 92' and three Kansas elite lines, KS030887K-6, KS09H19-2-3, and KS10HW78-1-1. Effectiveness of the RNAi construct has been evaluated using Real-time PCR. Results show up to 18-fold reduction in viral titer in the transgenic lines, the F_1 cross and the BC_1F_1 when

compared to control plants. This research provides the first evidence that a single host silenced transgene can provide resistance to multiple viruses.

Introduction

Wheat (*Triticum aestivum* L.), like all plants, contains the eukaryotic initiation factor (eIF) complex, which functions to aid in the recruitment of mRNA to the ribosome in order to complete translation (Gringas *et al*., 1999). The eIF4F complex actively recruits ribosomes and cellular mRNAs to initiate protein synthesis and this complex has multiple subunits. eIF4E and eIF4F subunits bind the 5'methyl guanosine cap of mRNAs. An RNA helicase, eIF4A, will unwind the mRNA 5' untranslated region (UTR) to facilitate the ribosome binding. Next, scaffold protein eIF4G facilitates an interaction with other translation machinery components (Gingras *et al*., 1999). Several isoforms of the components of the eIF complex also exist. eIF(iso)4F contains the isoforms eIF(iso)4E-2 and eIF(iso)4G (Browning, 2004). Beyond their normal function in plants, eIF's have also been found to be key determinants of the interactions of numerous RNA viruses and their plant hosts (Robaglia and Caranta, 2006).

Several viruses, from diverse families, use the eIF complex for cell-to-cell movement, translation, and replication (Urcuqui-Inchima *et al*., 2001). Viruses affecting plants, including potyviruses, lack the necessary proteins to complete their lifecycle; therefore, they are required to use host complexes. During potyviral infection, the eIF complex has been indicated to have direct interaction with the VPg, which is attached covalently to the end of the viral RNA (Leonard *et al*., 2000). The VPg mimics the 5' cap of eukaryotic mRNA in order to facilitate replication of the viral genome and interacting with the host cap binding protein, eIF4E (Leonard *et al*., 2000). This interaction has been shown to be required for successful infection (Thivierge *et al*., 2005). Direct interaction of the VPg and eIF4G has been confirmed to be required for

infection (Diaz-Pendon *et al*., 2004). eIF4G is also involved in the cap-independent translation of certain viruses (Nicaise *et al*., 2007).

In plants, post-transcriptional gene silencing (PTGS), or RNA interference (RNAi) is used to regulate gene expression. Double-stranded mRNA is regarded as foreign, which targets the mRNA for degradation (Poogin *et al*., 2001). RNAi has been hypothesized to be a mechanism of viral defense as well, and the use of transgenes that form hairpins have offered improvements in transgenic virus resistance (Waterhouse *et al*., 1998). Many of these have been based on virus sequences (Kalantidis *et al*., 2002; Di Nicola-Negri *et al*., 2005; Tougou *et al*., 2006; Fahim *et al*. 2010; Cruz *et al*., 2014). Faced with dsRNA, DICER-like enzymes process the dsRNAs into 21-25 nucleotide small interfering RNA (siRNA). Those siRNAs are incorporated in the RNA-induced silencing complex (RISC), which then leads to the degradation of any target mRNA with homology (Hammond *et al*., 2001; Campbell and Choy, 2005; Ruiz-Ferrer and Voinnet Olivier, 2007). This method has been used in wheat against *Wheat streak mosaic virus* (WSMV) and *Triticum mosaic virus* (TriMV), utilizing hairpins derived from the viral gene WSMV *NIa* (Fahim *et al*., 2010) and the coat protein (CP) gene from both WSMV and TriMV (Cruz *et al*., 2014; Rupp, unpublished). We hypothesized that with knockdown of one component of the eIF complex, the virus will not be able to replicate. The objective of this work was to utilize a RNAi hairpin containing a portion of the gene sequence derived from either of two interacting proteins involved in viral replication, eIF(iso)4E-2 and eIF4G to generate resistance to WSMV and TriMV, as well as other possible RNA viruses. Additionally, evaluation of the stability of this transgene over multiple generations was performed.

Materials and Methods

Cloning and plasmid construction

Primers were designed from the sequence of wheat eukaryotic initiation factor (iso) 4E-2 (GenBank Accession #WHT l F4F28A) and wheat eukaryotic initiation factor G (GenBank Accession #EF190330.1) using Integrated DNA Technologies, Primer Quest (www.idtdna.com). For the eIF(iso)4E-2, a 298 bp segment was selected from the forward primer sequence 5' CACCCGCAAATGGAGGCAAATGGACTGT and the reverse primer sequence TCCACCTCTGCTTGGTTTCTGACT . For eIF4G, a 517 bp sequence was selected with the forward primer 5'-CACCTCAGCAGCACCATTGGTATCTCCA and the reverse primer 5'-GCTCGGAGCATTCAACCTCCTCAA (Table 5.1). In order to ensure directional cloning of the PCR fragment into the entry vector, CACC was added to the 5' end of both forward primers for use with pENTR-D/TOPO (Life Technologies, Carlsbad, CA). Total RNA was extracted from wheat by collecting 100 mg of 'Bobwhite' leaf tissue. Tissue was ground in liquid nitrogen. Tissue was homogenized with 1 ml of TRIZOL® (Life Technologies, Carlsbad, CA). Samples were incubated at room temperature for five minutes. Two hundred µl of cholorform was added to each sample. Samples were vortexed for 30 seconds, then centrifuged at 10,000 x g in an 5415 C Eppendorf centrifuge at 4°C. RNA was precipitated with 500 µl isopropyl alcohol. The precipitate was washed twice with 500 µl of 70% EtOH before resuspension in 40 µl of diethyl pyrocarbonate (DEPC)-treated water. The RNA was quantified using a NanoDrop spectrophotometer (NanoDrop Technologies, Rockland, DE). One µg of total RNA was denatured at 65°C for 15 minutes, followed by placement on ice. MgCl2, (25 mM), 1X reverse transcription buffer (10mM Tris-HCL pH 9.0, 50mM KCl, 0.1% Triton® X-100), 2mM of each dNTP, 1U/µl recombinant RNasin ribonuclease inhibitor (Promega, Madison, WI), 1 U AMV

reverse transcriptase (NEB, Ipswich, MA) and 0.5 μg random primers/μg RNA (Promega, Madison, WI) was added to each tube. Following a 10 minute incubation at room temperature, samples were held at 42°C for 2 hours. Samples were then heated to 95°C for 5 minutes and transferred to ice. cDNA was used as the template for the eIF(iso)4E-2 and eIF4G primers described above. PCR products were purified using a QIAquick PCR Purification kit (Qiagen, Valencia, CA) following the manufacturers protocol. The purified PCR products were sub-cloned into the entry vector pENTER-D/TOPO following manufacturers instructions.

eIF(iso)4E-2 and eIF4G fragments were independently cloned into the pANDA-mini vector (Miki and Shimamoto, 2004). Plasmids were transformed in JM109 competent *E. coli* (Promega, Madison, WI). Plasmid purification was performed using an E.Z.N.A Plasmid Mini Kit (Omega Bio-tek, Norcross, GA). Insertional orientation was confirmed via Sanger sequencing (Kansas State University DNA Sequencing and Genotyping Facility.)

Plant transformation and culture

pANDA-mini expression vectors eIF4G and eIF(iso)4E-2 were co-bombarded with the expression vector pAHC20 (Christenson and Quail, 1996), which contains the *bar* gene for glufosinate resistance. Five independent biolistic experiments were completed, each using 180 total wheat calli. Immature seeds, 10 days post anthesis, were collected from the wheat cultivar 'Bobwhite' (CIMMYT, 1984). Seeds were surface sterilized in 20% v/v sodium hypochlorite (6%) and 0.04% v/v Tween-20. Excised embryos were then placed on callus induction medium, CM4 (Zhou *et al.*, 1995) in the dark at ambient temperature. One week later, organized callus tissue was selected for particle gun bombardment. Selected callus tissue was transferred onto fresh medium and underwent air drying to plasmolyze the cells for 1 hour in a laminar flow

cabinet. Biolistic particle delivery system, media usage, and tissue culture was as described by Anand *et al*., (2003a, b).

Molecular Characterization

Following selection and regeneration, plantlets that formed sufficient shoot and roots were transferred to peat pots, placed in a transparent box in a growth chamber at 18°C with high humidity conditions and over the next few days the box was slowly opened to acclimated the plants to lower humidity levels. Approximately one week after transplanting the plants were screened for Liberty™ resistance. Testing was done by painting a 0.2% v/v Liberty™ (glufosinate) solution (AgroEvo USA, Wilmington, DE) onto the surface of a single leaf using a small cotton tipped applicator. DNA was isolated from putative glufosinate resistant plants displaying an absence of necrosis. PCR was used for the detection of the *bar* gene and eIF constructs. Genomic DNA (gDNA) was isolated from approximately 10-mg leaf tissue using an E.Z.N.A. Plant DNA Kit (Omega Bio-tek, Norcross, GA). Two hundred ng of high quality gDNA was used in each PCR reaction. Each PCR reaction contained 10X PCR Buffer (Sigma Life Sciences, St. Louis, MO), $MgCl_2$ solution (25 mM) (Sigma Life Sciences, St. Louis, MO), 0.8 mM dNTPs, 10 pmol of each primer (Table 1), 1.25 U *Taq* DNA polymerase (Sigma Life Sciences), and 200 ng of high-quality template DNA. Amplification conditions were 5 min at 92°C; 30 cycles of 30 s at 92°C, 40 s at 60°C, and 60 s at 72°C; and a final extension at 72°C for 10 min. PCR products were run on 0.8% agarose gel containing 0.033ng/mL ethidium bromide in an electrophoresis box with 1X TAE buffer (50X 242 g TRIS base, 57.1 ml acetic acid, 100 mL 0.5 M EDTA pH 8.5), at 125 V for 20 minutes. Gels were visualized with a UV light box and photographed with a digital camera and Kodak 1D image analysis software. A high-throughput method was used on all subsequent generations. Leaf tissue was collected (3–6 cm^2)

from each plant and placed in 96-1.1mL collection racks (USA Scientific, Ocala, FL). Each tube contained one 3.96 mm steel bead (Abbott Ball Company, West Hartford, CT). Samples were flash-frozen in liquid nitrogen and stored at -80°C. Genomic DNA was isolated using BioSprint 96 DNA Plant Kits (Cat. No. 941558) following the manufacturer's instructions (Qiagen, Valencia, CA), resuspended in ddH_2O and stored in a -20°C.

Transgene expression was determined by extracting total RNA and deriving single-stranded complementary DNA (cDNA) by reverse transcription (catalog no. A3500, Promega). cDNA was used as a template for PCR reactions. Expression of the *gus* linker was determined by using *gus* sense and antisense primers (Table 5.1) to amplify a 636-bp fragment. Another reaction determined the presence and replication of viral RNA by PCR of the cylindrical inclusion (CI) gene fragment of either WSMV or TriMV or both. Genomic DNA presence in the cDNA was determined using α-tubulin primers (Li *et al*. 2005). Data from plants from the same event were pooled and tested using a chi-square test. Southern analyses were performed as in Faris *et al*. (2000).

Real-time RT-PCR (qPCR) was used to analyze the levels of viral titer and relative gene expression of the target genes. qPCR was performed on inoculated transgenic samples, crosses, backcrosses, parental lines and inoculated and mock inoculated 'Bobwhite.' One µg of total RNA was used to complete first strand synthesis with random hexamers, followed by reverse transcription with Superscript II (Invitrogen, Carlsbad, CA) according to the manufacturer's recommendations. Primers for qPCR were designed from the gene sequence of *Triticum mosaic virus* NIb (NCBI Reference Sequence: NC_012799.1) and *Wheat streak mosaic virus* NIb (NCBI Reference Sequence: NP_734273.1) and used to assess titer differences. iQ SYBR Green Supermix for iCycler (BioRad, La Jolla, CA) was used for all reactions. Three technical

replicates of each biological replicate were performed for each corresponding reaction. Primer efficiencies were tested prior to experimental use. Primers with efficiencies within the range of 90-110% were considered appropriate for use in experiments. The resulting Cq value for the target was subtracted from the Cq value of the internal reference gene Actin (Table 5.1).

RNA detection via Northern blotting was performed. Total RNA was extracted from 1g of leaf tissue using TRIZOL. Low molecular weight RNA was obtained using *mir*Vana miRNA extraction kit (Ambion Catalog number: AM1560) according to the manufacturers instructions. Total RNA was bound to a column resin and washed. Small RNA was selectively eluted by the addition of 1 ml of buffer QRW2 (50mM MOPS pH 7.0, 750 mM NaCl, and 15% ethanol). The precipitation of low molecular weight RNAs was made by the addition of 750 µl of ice-cold isopropanol and centrifugation at 12,000 x *g* for 30 min at 4°C. Low molecular weight RNAs were washed with 75% ethanol, air dried, and resuspended in 40 µl of DEPC water.

Low molecular weight RNA was separated using electrophoresis on 15% polyacrylamide gel (30% acrylamide 5ml, urea 4.2g, 10XTBE 0.5ml, 10% 80µl and TEMED 6 µl). Samples were denatured by adding one volume of deionized formamide and heated at 65°C for 5 min then, immediately transferred to ice for 5 min. Single stranded RNA loading dye was added and the mixture was loaded in to the gel. Two µl of the specific primers for the *gus* segment was used as a positive control. Gels were run for one hour at 100 volts.

siRNAs were transferred onto N^+ membrane (GE Healthcare, Buckinghamshire, UK) by electroblotting, at 80 volts for one hour, using 0.5X TBE as transfer buffer. The membrane was rinsed with 2X SSC with 0.1% SDS for 2 min; UV cross-linked and air-dried overnight. For pre-hybridization and hybridization, 8 ml of 65°C pre-warmed ULTRAhyb-oligo buffer (Ambion, Austin, TX) was used. The *gus* linker sequence was used as a probe in order to detect both

RNAi constructs. PCR products were amplified using *gus* sense and *gus* antisense primers. Following amplification, PCR product underwent PCR cleanup using QIAquick PCR purification kit (Qiagen), radioactively labeled, denatured at 95°C min for 10 min and then added into the hybridization tube. Hybridization was carried out at 42°C overnight in a rotating hybridization oven. Hybridization buffer was discarded and the membrane was washed with 1X SSC, 0.8% SDS for 15 min at 42°C and 1X SSC, 0.8% SDS for 15 at 42°C. The membrane was placed in a cassette and exposed to a phosphor image screen for two days, then scanned with the Storm ™840.

Genetic crosses

Crosses were made between transgenic wheat and the winter wheat cultivar 'Karl 92,' (Kansas State University, 1992) as well as three Kansas elite lines provided by Dr. Allan Fritz, (Kansas State University), KS10HW78-1-1, KS09H19-2-3, and KS030887K-6. 'Karl 92' is considered susceptible to *Wheat streak mosaic virus* and very susceptible to *Triticum mosaic virus*. In the initial cross, the transgenic lines served as the female parent, while 'Karl 92' served as the male parent. Reciprocal crosses were also made. Resulting crosses were tested via PCR, RT-PCR and under challenge with both viruses individually and with co-infection. Tested lines were followed through to two backcrosses to the respective recurrent parent and tested as described above.

Bioassays

Plants were inoculated by applying a light dusting of carborundum and 40 µl of infected plant sap (100 mg of desiccated infected leaf tissue in 100 µl 0.02 M sodium phosphate buffer, pH 7.4) to the second or third leaf of 2-wk-old seedlings. The leaf was then pinched between the thumb and forefinger and the inoculum was pulled down the length of the leaf a minimum of ten

times. Plants were inoculated a second time 14 days later to ensure infection. Inoculated plants were grown in a growth chamber with 16-h day/8-h night, 500 μE/m^2/s light intensity, at 19°C. The T_1 generation was challenged with WSMV only. Leaf samples were taken 14–21 d post-inoculation. WSMV virus presence was determined by double antibody sandwich enzyme-linked immunosorbent assay (DAS- ELISA) according to the manufacturer's protocol (Agdia, Elkhart, IN). The T_2 generation was challenged with TriMV only. Indirect ELISA was performed as in chapter 4.

Absorbance was measured at 405 nm using a Biotek EL-800 Absorbance Reader (Biotek, Highland Park, Winooski, VT). Samples from inoculated and mock-inoculated non-transgenic wheat and transgenic wheat were used in the assays. Adjustments were made to the raw ELISA readings in order to standardize across multiple plates using the following formula: (sample – blank)/(negative control – blank) and samples with an adjusted value of 2.0 or above were considered positive for the virus (Zhang *et al*., 2011). Beginning in the T_3 generation all plants were challenged with co-infection of WSMV and TriMV. Plants were inoculated at the three-leaf stage with WSMV. The following day plants were inoculated with TriMV. This sequence was repeated 14 days later. Two weeks following the second inoculation, plants were scored for virus symptoms, and two separate 2.54 cm samples of leaf tissue were taken for testing with ELISA.

T_3, T_4 and T_5 plants were measured for individual height. Plants were allowed to reach full maturity. Seed was harvested from each plant and weighed. Samples from transgenic material, nontransgenic inoculated wheat, and mock-inoculated samples were used in the assays, as well.

Temperature Bioassays

Three replicates of 5 plants of each from T_4 lines and T_5 lines originating from the three original eIF4(iso)E-2 lines and the four originating from the eIF4G events were grown in Conviron growth chambers at three temperature regimes (18°C, 20°C and 23°C) at 16-h day/8-h night, 500 μE/m^2/s light intensity. Plants were inoculated with both WSMV and TriMV, sampled and analyzed as described above.

Aphid screening

Schizaphis graminum Rondani samples were field collected from the Kansas State University North farm off plants exhibiting the symptoms of *Barley yellow dwarf virus*. The aphid colony was maintained on the cultivar 'Tam 107' in under growth chamber conditions of 16-h day/8-h night, 500 μE/m^2/s light intensity, at 18°C. Aphids were supplied with fresh plant material every 14 days. Presence of BYDV was confirmed using a double antibody sandwich enzyme-linked immunosorbent assay (DAS- ELISA) according to the manufacturer's protocol (Agdia, Elkhart, IN). The Kansas State University Virus-Vector lab confirmed the presence of three strains carried by the population: BYDV-PAV, BYDV-MAV, and BYDV-RPV (data not shown). In an initial screen, remnant T_2 seed was bulked for a T_3 screen with the virus. Six seed of each transgenic line was planted in an 8" x 8" tray in a completely randomized design. At the two-leaf stage, ten viruliferous aphids were transferred to each plant. Aphids and plants were maintained in a glass cage, with a fine mesh top. Aphids were given a four-day inoculation access period. Planting trays were then sprayed with the pesticide Marathon (imidocloprid) and allowed to continue to grow for two weeks. Plants were then sampled and scored individually. Samples were taken for PCR, RT-PCR and ELISA at this time. Plants were categorized as both having and expressing the transgene, and the resistance response was recorded. T_4 generation

transgenic lines were grown for seed increase. Five replicated trials of each event of the T_5 lines were challenged with the BYDV viruliferous aphids by placing ten aphids on each plant in growth chambers. Each plant was sampled for PCR, RT-PCR and ELISA individually.

Soilborne wheat mosaic virus assay

Soil, infested with *Polymyxa graminus* Ledingham was field collected from the Rocky Ford Experiment station, Kansas State University. Samples were allocated into 8" x 8" metal baking pans containing holes drilled in the bottom, and placed on trays. Seeds of the *Soilborne wheat mosaic virus* (SbWMV) susceptible cultivar 'Ernie' (University of Missouri, 1994) were grown in infested soil. Following seed emergence, soil was repeatedly surface flooded for five consectutive days. After the completion of the flooding event, plants were allowed to grow for 20 days at 16-h day/8-h night, 500 $\mu E/m^2/s$ light intensity, at 18°C/16°C.

At 20 days post flooding, leaf samples were taken and analyzed with DAS-ELISA for the presence of SbWMV following manufacturers instructions (Agdia, Elkhart, IN). Following confirmation of SbWMV presence, bulked T_2 seed was planted, 25 seeds of each line chosen, into pans containing infested soil and grown as described above along with 'Ernie' and resistant cultivar 'Fuller' (Kansas State University, 2004). After flooding, plants were allowed to grow normally for 20 days. At 20 days post flooding, leaf samples were taken and analyzed with DAS-ELISA for the presence of SbWMV.

Results

Portions of eIF(iso)4E-2 and eIF4G genes of wheat were independently amplified and cloned into pANDA-mini vector, which consisted of a sense and antisense copy of each sequence, separated by the *gus* linker sequence. Five independent biolistic transformation experiments were performed using a total of 900 wheat callus explants and 119 eIF(iso)4E-2 and

72 eIF4G putative transgenic plants were regenerated. These were screened using the herbicide glufosinate. Twenty-two plants were determined to putatively contain the *bar* gene, and underwent PCR analyses using gDNA. Seven plants were found to contain the complete hairpin construct; three from eIF(iso)4E-2 (Events 1550, 1814, and 1822) and four from eIF4G (Table 5.1) (Figure 5.2). Seeds of the T_0 plants were independently collected. Twenty-four seeds of each of the T_0 lines of the T_1 generation were planted.

In the T_1 generation, each of the lines were found to express the transgene to varying degrees. Segregation in these lines fit a 3:1 ratio, suggesting integration at a single locus. These plants were grown and challenged with WSMV. At 21 days after the second inoculation, samples from the youngest leaves were taken for molecular analysis and symptoms were recorded. Each of the seven events produced plants with high levels of resistance to the virus. Each tiller of the T_0 had been harvested separately, and given a letter designation for selection records, while each plant of the T_1 generation and beyond was harvested separately.

For the T_2 generation, 240 plants originating from the initial 7 events and derived by single seed descent from resistant plants in the T_1 generation were grown and given an additional letter designation. One hundred seventy-six plants were shown to contain and express the transgene (Figure 5.3). Plants were inoculated with TriMV. At 21 dpi, samples from the youngest leaves were taken for molecular analysis and symptoms were recorded. Each of the seven events produced plants with high levels of resistance to the virus. Viral RNA could be detected in plants with symptoms and negative-control non-transgenic plants, transgenic non-GOI plants, and control varieties showed 100% virus incidence. Plants were rated on a (1-9) scale, with 9 being extremely susceptible. Single seed selection was performed based on the criteria that plants contained and expressed the transgene, had a rating of 4 or below on the

phenotypic rating scale, and had ELISA values comparable to that of buffer only control. The *gus* linker sequence could be detected via Northern blot analysis in plants from all seven events (Figure 5.5)

In the T_3 generation, 214 plants originating from the three initial events of eIF(iso)4E-2 and selected through single seed descent from top performers in the T_2 generation were grown. Each of these individual plants was given an additional letter designation. One hundred eighty-six were found to contain the transgene of the eIF(iso)4E-2 lines and 170 were found to be resistant to co-infection of WSMV and TriMV. Two hundred seventy plants of eIF4G were grown from the four initial events, and single seed descended from top performers in the T_2 generation. Two hundred forty-one were found to contain the transgene. All 241 plants containing the eIF4G transgene were considered resistant to co-infection. Single seed selection was performed to generate a T_4 population consisting of 1,387 individual plants, 554 originating from the three eIF(iso)4E-2 events, and the remaining from the eIF4G events. Seven hundred ninety plants contained and expessed the transgene, and all were considered resistant to co-infection of WSMV and TriMV.

All plants in the T_3, T_4 and T_5 generation were measured for height and harvest weight individually. Analysis revealed that there was no statistical difference in height or harvest weight between the transgenic lines containing the transgene and considered resistant to co-infection of WSMV and TriMV and the non-inoculated controls. However, there was a statistical difference between the susceptible controls and those non-resistant transgenic lines either no longer containing/or expressing the transgene ($p<0.05$) (Figure 5.5).

T_5 analysis was performed as in previous generations, but also included replicated temperature trials at 18°C, 20°C, and 23°C. T_5 plants used originated from the original seven

events and were directly descended from top performing plants found the in the T_4 generation, which were then pooled. T_5 transgenic plants from T_4 pooled lines, representing event families were used in 3 replicates at above temperatures, using approximately 5 seeds each replicate at each temperature. The varieties 'RonL', 'Tomahawk' and 'Bobwhite' and 'Karl 92' served as susceptible controls. Mock-inoculated 'Bobwhite' was also used. Each plant was subjected to co-infection as described above. Control plants had a 100% disease incidence in every replicate at each temperature regime (Table 5.2).

Several lines in the T_5 generation were chosen for crossing with the winter wheat variety, 'Karl 92' and to three KS elite varieties; KS10HW78-1-1, KS09H19-2-3, KS030887K-6. The reciprocal cross was also made from the same plant. In total, 24 crosses were made from lines 1550, 1742, 1673, 1755, 1814, 1822, and 1830 to 'Karl 92'. The F_1 seed was recovered from all crosses. These crosses were then grown, 5 seeds each and challenged with the virus. All F_1 crosses were considered resistant. Additional seed from the F_1 cross was then used for a backcross with the recurrent parent. These BC_1F_1's were grown, underwent PCR confirmation for transgene presence, RT-PCR to confirm transgene expression and challenged with co-infection using WSMV and TriMV. All BC_1F_1's were resistant to the viruses. An experiment was performed in three replicates containing five each of mock-inoculated Bobwhite, inoculated Bobwhite, and seeds originating from each cross. These plants were grown under greenhouse conditions and challenged with both viruses. Inoculated 'Bobwhite' and 'Karl 92' showed 100% disease incidence, received a '9' severity rating, were extremely stunted and produced little to no seed. Each of the transgenic lines, transgenic crosses and mock-inoculated 'Bobwhite' showed no visible symptoms, the CP was undetectable with ELISA, and no viral RNA was detected via RT-PCR. Plants were then analyzed using real-time PCR. Values of relative expression of the

viral gene WSMV *NIb* and TriMV *NIb* representing the level of viral titer were calculated using the ΔΔCt method. The results are displayed in Figure 5.6. Southern analyses revealed multiple copies of the transgene (Fig. 5.7).

Resistant plants were selected from the T_5 families and grown for the T_6 generation and the progeny were again resistant. Presence of the transgene was determined by PCR was evaluated and following viral challenge plants were evaluated via ELISA. Stable resistance was evident. Two hundred sixty plants were grown from the T_5 pooled event families. All plants were found to contain and express the transgene. All plants were considered resistant when challenged with co-infection of WSMV and TriMV.

These two transgenes were tested in a five replicate bioassay in which they were challenged with an aphid population that is infected with *Barley yellow dwarf virus* (BYDV). Fifteen seeds originating from each of the original seven events, plus controls were challenged with viruliferous aphids. Plants were grown to the two-leaf stage. Ten viruliferous aphids were placed each plant and an inoculation access period of four days was allowed. Following a pesticide treatment, plants were allowed to grow for three additional weeks to test for resistance to BYDV. In total, 202 plants from T_5 pooled event families were found with significant levels of resistance based on ELISA (Figure 5.8).

Lines were also tested in a preliminary screen in which they were grown in soil infested with *Soilborne wheat mosaic virus* (SbWMV). Plants were grown from each of the original seven events, as well as susceptible ('Ernie') and resistant ('Fuller') controls. Plants were tested using ELISA (Table 5.3). The T_3 generation derived by T_2 bulk contained null segregants, due to the lack of selection of GOI positive plants in the T_2 generation. Those plants found to contain the GOI, were found to express the transgene to varying degrees, from very low to very

high expression via RT-PCR. However, resistance to SbWMVwas found in the majority of those plants found to contain and express the transgenes from both eIF(iso)4E-2 and eIF4G.

Discussion

The importance of eIF4E has become evident during infection of viral species in the family *Potyviridae*. In Potyviruses, the VPg physically interacts with eIF4E or its isoforms, in addition to the poly(A)-binding protein (PABP) (Léonard *et al*., 2000, 2004; Schaad *et al*., 2000; Wittmann *et al*., 1997). This interaction is required for both the virus infection and completion of its lifecycle (Duprat *et al*., 2002; Lellis *et al*., 2002). It has been proposed that inhibition of cellular mRNA translation might be achieved by the competition between VPg and cap for a particular eIF4E factor (Khan *et al*., 2008; Léonard *et al*., 2000). Mutant alleles of eIF4E showing impairment of the interaction with VPg are responsible for observed resistances to potyviral infections (Gao *et al*., 2004; Lellis *et al*., 2002). The central region of the VPg has been implicated in the interaction with eIF4E (Roudet-Tavert et al., 2007), and the VPg has been proposed as an avirulence factor in the potyvirus-plant interaction (Rajamaki and Valkonen 1999).

Potyviruses use the eIF4E isoforms as well. In *A. thaliana*, eIF(iso)4E but not eIF4E is required for successful viral colonization in the case of *Tobacco etch virus* (TEV) (Lellis *et al*., 2002). Disruption of the isoform, eIF(iso)4E in *Arabidopsis thaliana* by chemical or transposon mutagenesis resulted in complete or near-complete immunity to several Potyviruses, including *Turnip mosaic virus* (TuMV), *Lettuce mosaic virus*, and *Plum pox virus,* and. *Clover yellow vein virus* (ClYVV). ClYVV accumulated in leaves of mutant *Arabidopsis* plants lacking eIF(iso)4E, but not in mutants lacking eIF4E. TuMV replicated in mutant plants lacking eIF4E, but not in

mutants lacking eIF(iso)4E. This unique finding indicated that some of these factors interacted differently with certain viruses, which indicates selective requirement of certain elements of the complex during potyviral infection (Sato *et al*., 2005).

Following the description of the VPg as the avirulence factor for recessive resistance genes in various plants (Nicolas *et al*., 1997, Keller *et al*., 1998), it has been reported that the VPg of TuMV interacts with, but does not disrupt, the translation initiation complex eIF(iso)4E-eIF(iso)4G. The VPg was also found to interact with the eIF4(iso)4G. The interaction decreased the affinity of the translation initiation machinery for capped mRNAs (Plante *et al*., 2004). VPg inhibits host protein synthesis at early stages of the initiation complex formation through the inhibition of cap attachment to the initiation factor eIF4E (Grzela et al., 2006). The potyviral VPg has been shown to allow translational enhancement mediated by an internal ribosome entry site (IRES) in the 5'UTRs, which binds to eIF4G via an RNA pseudoknot (Zeenko and Gallie, 2005; Khan et al., 2009, Newburn and White, 2015). The activity of this IRES is further promoted by the presence of the 3′-poly(A) tail, indicating a level of cooperation between these atypical and conventional RNA elements (Gallie *et al*., 2001).

TEV VPg has been shown to interact with eIF4E or eIF(iso)4E (Gallie *et al*., 2001). eIF4E and eIF(iso)4E has been shown to be a determining factor is plant host susceptibility to TEV (Estevan *et al*., 2014). A Mexican isolate of TEV depends on eIF(iso)4E for its systemic spread. The absence or overexpression of eIF(iso)4E did not affect viral translation, and replication was still observed even in the absence of eIF(iso)4E. The systemic spread was completely eliminated in the null mutant. The VPg precursor NIa was found in co-immunoprecipitated complexes with both, eIF(iso)4E and eIF4E. However, the viral coat protein (CP) was only present in the eIF(iso)4E complexes. Since both the VPg and the CP

proteins are needed for systemic spread, this indicated eIF(iso)4E had a role in the movement of TEV as well (Contreras *et al*., 2012)

Eukaryotic initiation factors are involved in luteovirus translation as well. The 3' cap-independent translation element (BTE) of *Barley yellow dwarf virus* RNA involves long-distance base pairing with the 5'-untranslated region (UTR). This requires the recruitment of eIF4F. It has also been shown as well as BTE interacts specifically with the cap-binding initiation factor complexes eIF4F and eIFiso4F in a wheat germ extract. Full-length eIF4G and the C-terminal half of eIF4G lacking the eIF4E binding site stimulated translation to 70% of the level obtained with eIF4F, indicating a minor role for the cap-binding protein, eIF4E. In wheat germ extract, eIF4G alone restored translation nearly as much as eIF4F, while addition of eIF4E alone had no effect. The BTE bound eIF4G and eIF4F with high affinity, but very weakly to eIF4E. This was consistent with the model in which eIF4F is delivered to the 5' UTR by the BTE, and they show that eIF4G, but not eIF4E, plays a major role in this novel mechanism of cap-independent translation (Treder *et al*., 2008).

Little is known about what host proteins interact with *Soil borne wheat mosaic virus*, a furovirus. This is most likely a function of the continued durable dominant resistance gene available to the disease, *Sbm1* (Bass *et al*., 2006). Preliminary results in this study indicate that the possibility of resistance to SbWMV in all seven events, but will require replicated studies using lines that consistently contain and express their transgenes. Lack of resistance may have been due to low expression of the transgenes in individual plants, or possible occurences of transgene silencing. It is hypothesized some of the resistance mechanisms available for SbWMV are based on the blocking of systemic virus infections. This is supported by field trials during which SbWMV could not be detected visually, or by ELISA in above ground tissue, but could be

detected in roots (Budge *et al*., 2002). Similarly, resistance in soft white winter wheat to *Wheat spindle streak mosaic virus* (WSSMV, genus *Bymovirus*) has been suggested to restrict upward movement of the virus (Carroll *et al*., 2002; McGrann and Adams, 2004). Much work is required in order to determine how resistance functions, as well as the host proteins that interact with the virus.

This is the first report of two single host transgenes exhibiting resistance to multiple viruses. This is also only the third report of a the stability of these types of hairpin transgene functioning over many generations (six), the first being Cruz *et al*., 2014 studying transgenic wheat containing a hairpin derived from the gene sequence of the CP of *Wheat streak mosaic virus*, the second being transgenic wheat containing a hairpin derived from the gene sequence of the CP of *Triticum mosaic virus* (Rupp, *in preparation*). In this work, it is assumed that the hairpin sequence of eIF(iso)4E-2 and eIF4G is responsible for the resistance. The host gene knockdown is attributed to RNAi induction by the dsRNA. This may be due to the unavailability of host genes required to complete the viral lifecycle. This could also be due to the competition between the potyviral VPg and cap for a particular eIF factor. The method of targeting the CP has been shown to be effective in wheat using Potyviruses and gene sequences derived from the virus. In WSMV, Cruz *et al*., (2014) plant lines were carried into the T_5 and successful resistance was maintained utilizing a hairpin derived from the CP. Fahim *et al*. (2011), also targeting WSMV, was successful in generating wheat that maintained resistance to WSMV using a hairpin derived from the *NIa* gene through the T_2 generation. The use of hairpin constructs targeting potyviral CP's to generate resistance has also been reported in other species (Guo *et al*., 2015; Kertbundit *et al*., 2007; Krubphachaya *et al*., 2007; Hily *et al*., 2007; Tougou *et al*., 2006; Wang *et al*., 2000) confirming that this is a viable strategy in a variety plant species against a multitude

of viruses. In particular, the use of hairpin constructs has effectively been shown to increase efficiency of PTGS (Smith *et al*., 2000) in comparison to constructs bearing only sense or antisense. Furthermore, RNAi based gene silencing strategies in wheat for functional gene analysis has been shown to reduce the expression of targeted endogenous genes (Fu *et al*., 2007)

Figures and Tables

Primer name	Sequence (5"-3")	Product size	Description
eIF(iso)4E-2F	CACCCGCAAATGGAGGCAAATGGACTGT	298 bp	Amplification of eIF(iso)4E-2 fragment in RNAi vector
eIF(iso)4E-2R	TCCACCTCTGCTTGGTTTCTGACT		
eIF4GF	CACCTCAGCAGCACCATTGGTATCTCCA	517 bp	Amplification of eIF4G fragment in RNAi vector
eIF4GR	GCTCGGAGCATTCAACCTCCTCAA		
gus F1	CACGTAAGTCCGCATCTTCA	216 bp +GOI	Used with the gene specific primers to determine presence of GOI
gusR1	ATCTCTTTGATGTGCTGTGCC	353 bp+GOI	
gusR2	GTATCAGTGTGCATGGCTGG	154 bp +GOI	Used with specific CP primers to determine presence of GOI
gus Sense	CATGAAGATGCGGACTTCCG	636 bp	RT-PCR primers to establish gus linker expression
gus Antisense	ATCCACGCCGTATTCGG		
TriMV2-R	TCTGTTCCTGTGGTGAAAGCTGGT	408 bp	Used in RT PCR to determine virus presence in TriMV transgenic material
TriMV2-F	CGGCAGCAAATGGACTTGGATTGA		
WSMV-CIF	TCCAGGAATGGGCGTGTGATGATA	256 bp	Used in RT PCR to determine virus presence in TriMV transgenic material
WSMV-CIR	ACACTAGCATCTCTGCCGAGGTTT		
BarABR	CCTGCCTTCATACGCTATTTATTTGC	500 bp	Amplification of *bar* gene
UbiABF	CTTCAGCAGGTGGGTGTAGAGCGTG		
TubF	ATCTGTGCCTTGACCGTATCAGG	409 cDNA	RT- PCR primers internal control used to determine cDNA contamination
TubR	GACATCAACATTCAGAGCACCATC	500bp gDNA	
TriMV qNib-F	GCTGAGTTGAGACCGAAAGAA	135 bp	Real-time PCR primer to detect TriMV
TriMV qNib-R	GCC TGC CTG TGT AGC ATA AA		
WSMVNIaqbF	TGGACCGATCGGATTAAG	102 bp	Real-time PCR primer to detect WSMV
WSMVNIaqbR	CCGTAGAAGTGCCAGTAT		
Actin qa-F	GTTCTCAGTGGAGGTTCTA	113 bp	Real-time PCR primer to detect housekeeping gene
Actin qa-R	CTTTCAGGTGGTGCAATAA		

Table 5.1: Primers and descriptions used in this study

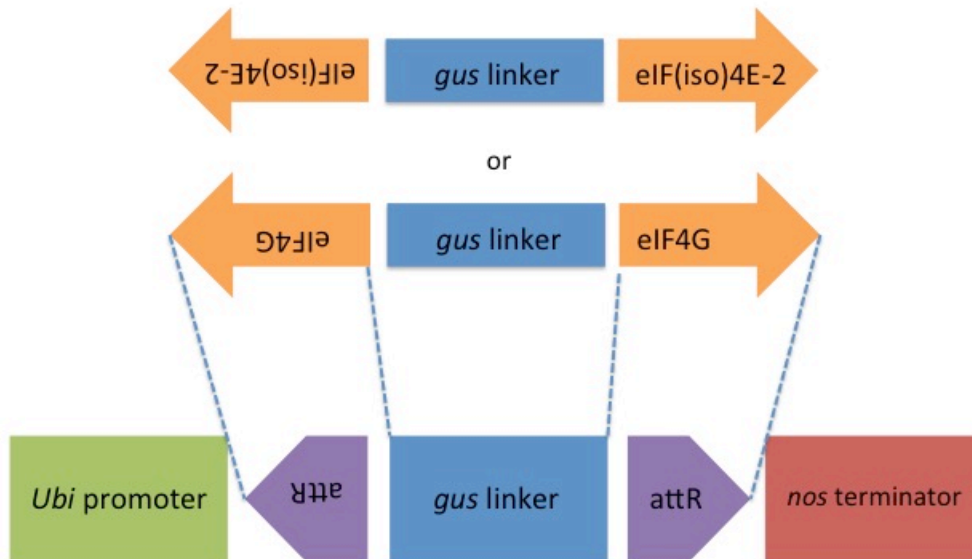


Figure 5.1: Schematic representation of the eIF(iso)4E-2 or eIF4G RNAi vector construction. pANDA-mini (Miki and Shimamoto 2004) vector with attR sites allow for the homologous recombination insertion of inverted sequences derived independently from eIF(iso)4E-2 and eIF4G flanking a gus linker (Cruz *et al*., 2014). When expressed in wheat, the inverted eIF sequences form a hairpin structure causing the sequence(s) to be targeted by the Dicer system**.**

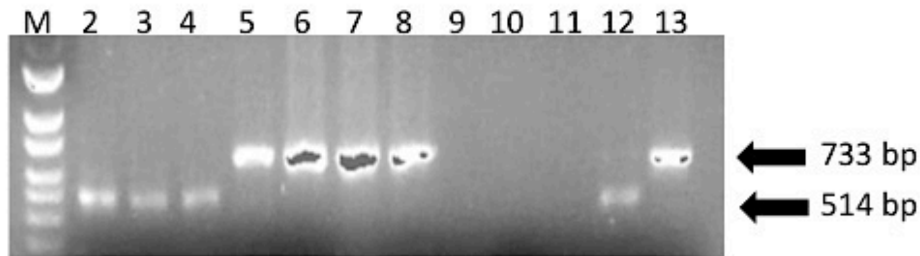


Figure 5.2: PCR analyses on gDNA of T_0 eIF RNAi transgenic gus forward (gus F1) and either eIF4(iso)E-2R or eIF4GR primers were used on genomic DNA. Each plant was resistant to application of glufosinate. Controls were non-transgenic Bobwhite (BW), water only and plasmid DNA for PCR positive control. Lane 1: Marker, Lane 2: Event 1550, Lane 3: Event 1814, Lane 4: Event 1822, Lane 5: Event 1673, Lane 6: Event 1742, Lane 7: Event 1755 Lane 8: Event 1830, Lane 9: Non-transgenic Bobwhite, Lane 10: Transgenic Non-GOI Bobwhite, Lane 11: H_20 Lane 12: eIF(iso)4E-2 plasmid, Lane 13: eIF4G plasmid.

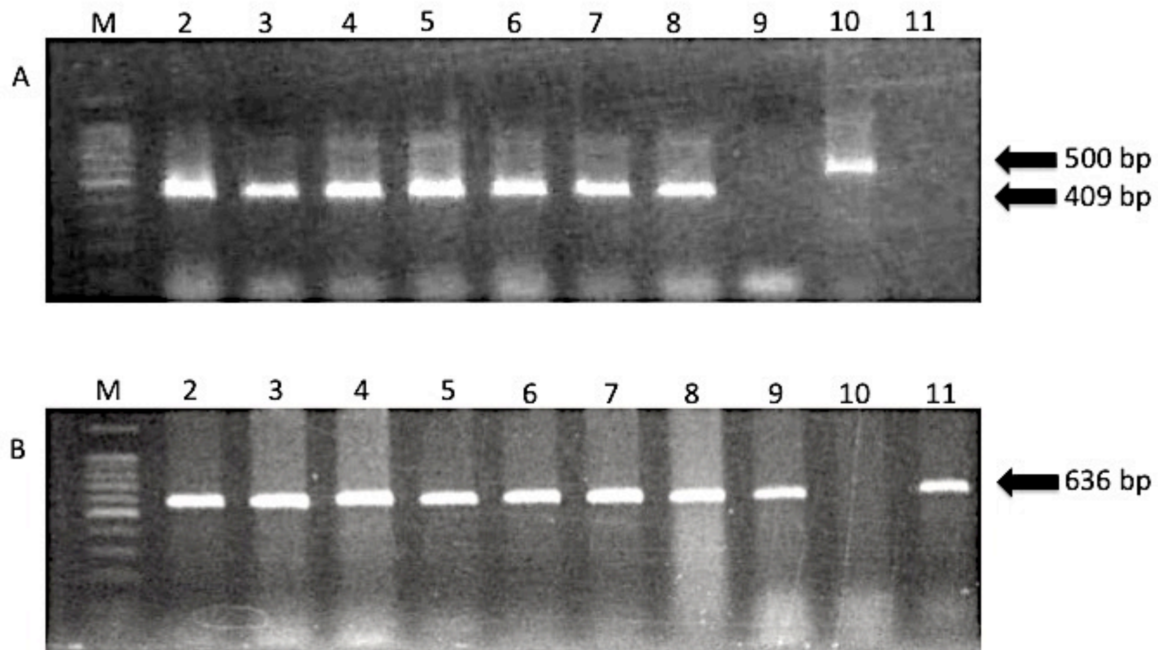


Figure 5.3: RT-PCR analyses on RNA of T_2 eIF RNAi transgenic wheat reconstituted by T_1 pooling. Panel A: Primers Tubulin Forward (Tub-F) and Tubulin Reverse (Tub-R) were used. Lane 1: Marker, Lane 2: Event 1550, Lane 3: Event 1814, Lane 4: Event 1822, Lane 5: Event 1673, Lane 6: Event 1742, Lane 7: Event 1755, Lane 8: Event 1830, Lane 9: eIF(iso)4E-2 plasmid, Lane 10: gDNA Transgenic Non-GOI Bobwhite, Lane 11: eIF4G plasmid; Panel B: Primers gus sense and gus antisense were used. Lane 1: Marker, Lane 2: Event 1550, Lane 3: Event 1814, Lane 4: Event 1822, Lane 5: Event 1673, Lane 6: Event 1742, Lane 7: Event 1755 Lane 8: Event 1830, Lane 9: eIF(iso)4E-2, Lane 10: gDNA Transgenic Non-GOI Bobwhite, Lane 11: eIF4G plasmid.

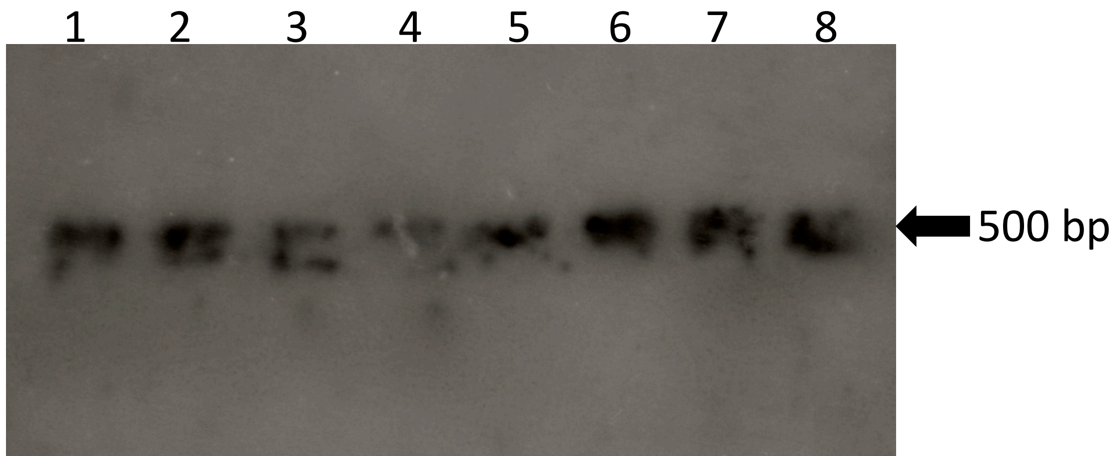


Figure 5.4: Detection of *gus* insert via Northern Blot Analysis of T_2 lines, by event. Lane 1: Event 1550, Lane 2: Event 1814, Lane 3: Event 1822, Lane 4: plasmid DNA, Lane 5: Event 1673, Lane 6: Event 1742, Lane 7:, Event 1755, Lane 8: Event 1830

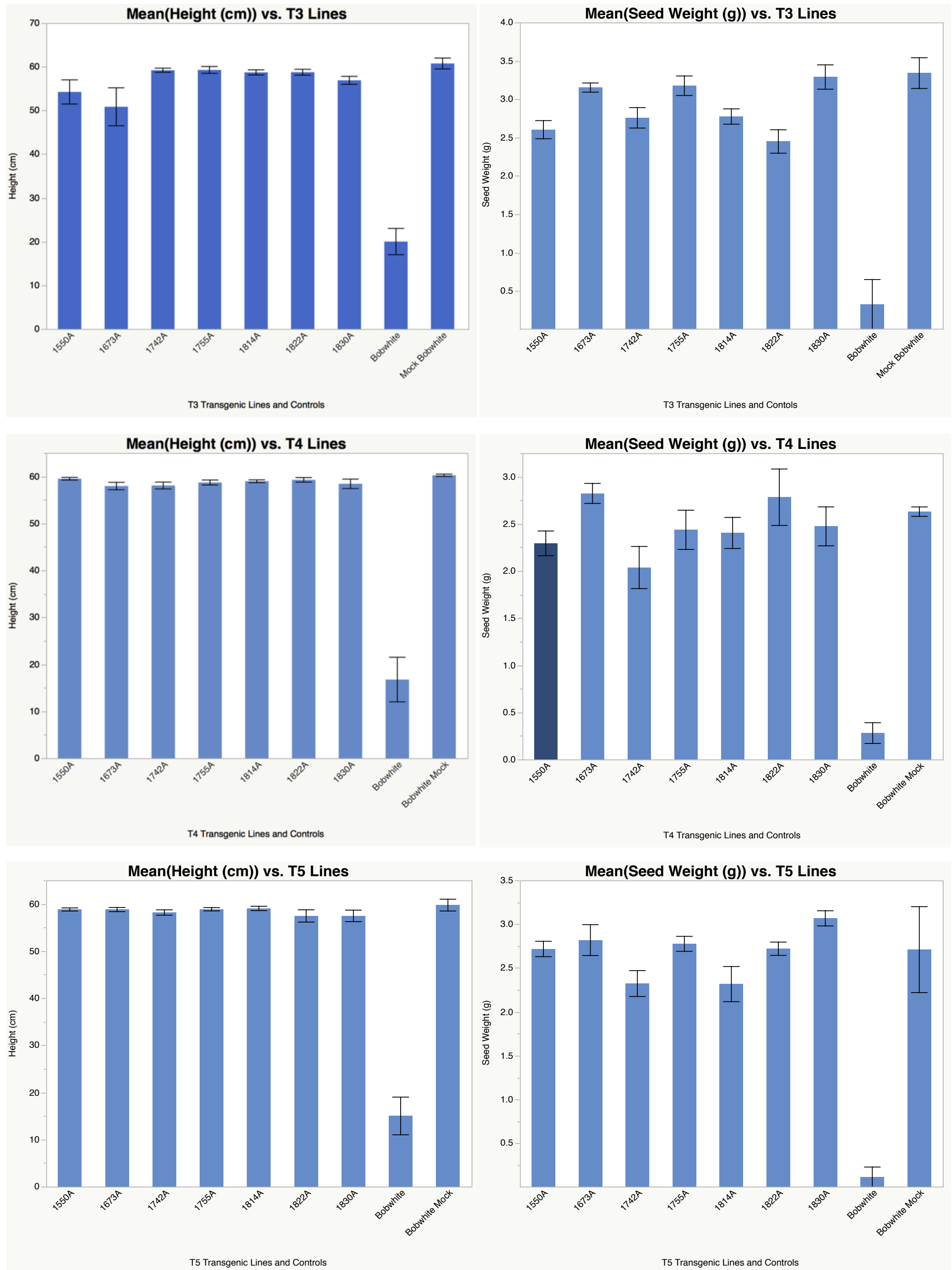
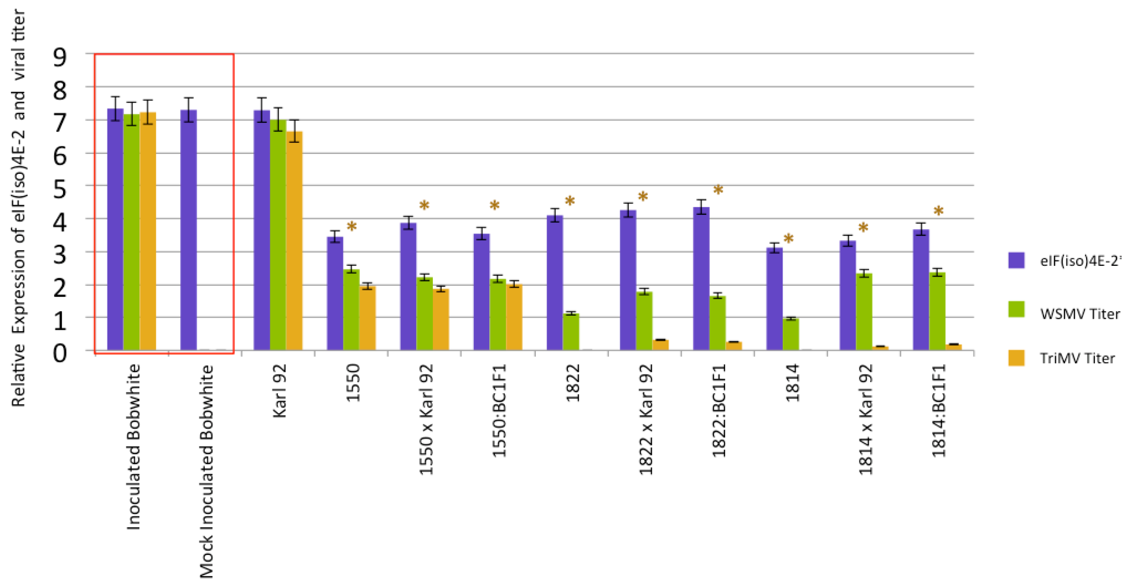


Figure 5.5: T_3 ,T_4, and T_5 Generation analyses of plant height (cm) and seed weight produced by individual plants (g) averaged among events challenged with virus. No statistical difference was found between the height or seed weight of transgenic lines and non-inoculated 'Bobwhite', while seed weight and height are statistically ($p<0.05$) different between inoculated controls, and those transgenics lacking the GOI and that are considered non-resistant.



*Indicates a p-value <0.05 when using Dunnett's Adjustment to compare with controls

Figure 5.6: Relative Expression of eIF(iso)4E-2, relative abundance of WSMV viral gene *NIb*, and TriMV viral gene *NIb*, in inoculated 'Bobwhite,' mock inoculated 'Bobwhite,' inoculated parental line 'Karl 92', and inoculated transgenic lines, F_1 crosses, and BC_1F_1's. Five plants were grown of each line and challenged with co-infection WSMV and TriMV in each of three experimental replicates. At forty-two days after the second inoculation, plants were sampled for analysis. Three biological replicates with three technical replicates were used in each experiment. * Indicates significance $p<0.05$ using Dunnett's Adjustment to compare to Inoculated Bobwhite when averaged over the three experimental replicates.

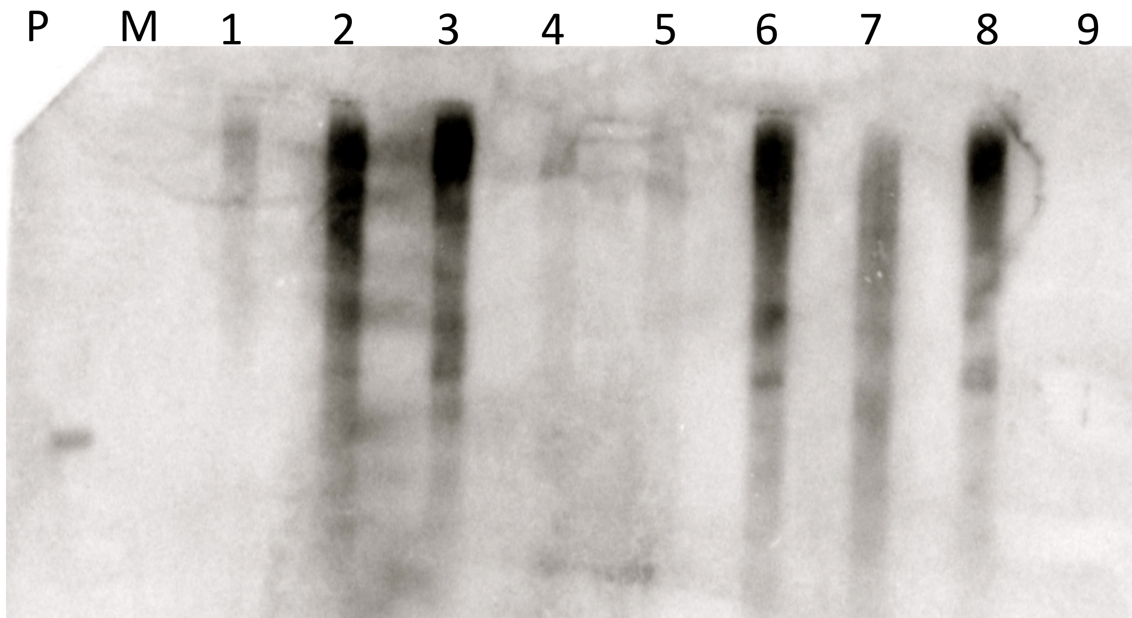


Figure 5.7: Southern Blot Analysis of T_5 lines, by event, probed with PCR segment from primers eIF4GR and *gus* R2. Digested with *KpnI*. Lane P: eIF4G RNAi plasmid, Lane M: 1 Kb plus marker, Lane 1: Event 1550. Lane 2: Event 1673, Lane 3: Event 1742, Lane 4: Event 1814, Lane 5: Non-transgenic Bobwhite, Lane 6: Event 1755, Lane 7: Event 1822, Lane 8: Event 1830, Lane 9: eIF(iso)4E-2 RNAi construct

Temperature Bioassays of T_5 eIF(iso)4E-2 and eIF4G Transgenic lines Co-infected with WSMV and TriMV									
eIF(iso)4E-2	**Rep 1**			**Rep 2**			**Rep 3**		
T_5 Lines	**18°C**	**20°C**	**23°C**	**18°C**	**20°C**	**23°C**	**18°C**	**20°C**	**23°C**
1550A-A-A-2.1	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
1550A-A-3-2.1	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
1550A-A-4-1.1	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
1550A-A-6-1.1	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
1550A-A-7-2.1	0/5	0/5	1/5	0/5	0/5	0/5	0/5	0/5	0/5
1550A-A-9-4.1	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	1/5
1814A-A-2-1.1	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
1814A-A-2-1.1	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
1814A-A-3-2.2	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
1814A-A-5-3.1	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
1822A-A-2-1.4	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
1822A-A-3-2.1	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
1822A-A-7-1.1	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
eIF4G	**Rep 1**			**Rep 2**			**Rep 3**		
T_5 Lines	**18°C**	**20°C**	**23°C**	**18°C**	**20°C**	**23°C**	**18°C**	**20°C**	**23°C**
1673A-A-1-1.1	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
1673A-A-11-1.1	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
1673A-A-2-4.1	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
1673A-A-3-2.1	0/5	0/5	2/5	0/5	0/5	0/5	0/5	0/5	0/5
1673A-A-5-2.1	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
1673A-A-9-1.1	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
1742A-A-1-1.1	0/5	0/5	0/5	0/5	0/5	1/5	0/5	0/5	0/5
1742A-A-4-4.1	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
1742A-A-6-2.1	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
1742A-A-7-1.1	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
1755A-A-1-2.1	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
1755A-A-4-1.1	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
1755A-A-5-2.2	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
1755A-A-6-4.1	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
1755A-A-7-1.1	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
1755A-A-8-1.3	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
1830A-A-1-1.1	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
1830A-A-6-1.2	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	1/5
1830A-A-7-4.2	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Control Plants	**Rep 1**			**Rep 2**			**Rep 3**		
Lines	**18°C**	**20°C**	**23°C**	**18°C**	**20°C**	**23°C**	**18°C**	**20°C**	**23°C**
NI Bobwhite	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
NT Bobwhite	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5
Non GOI Bobwhite	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5
Karl 92	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5
Tomahawk	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5
RonL	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5

Table 5.2: Temperature bioassays of T_5 eIF plants derived from single seed selection, grown in growth chambers at 18°C, 20°C and 23°C. NI Bobwhite: non-inoculated Bobwhite, NT Bobwhite: Non-transgenic inoculated Bobwhite, Non-GOI Bobwhite, Karl 92, Tomahawk and RonL served as controls.

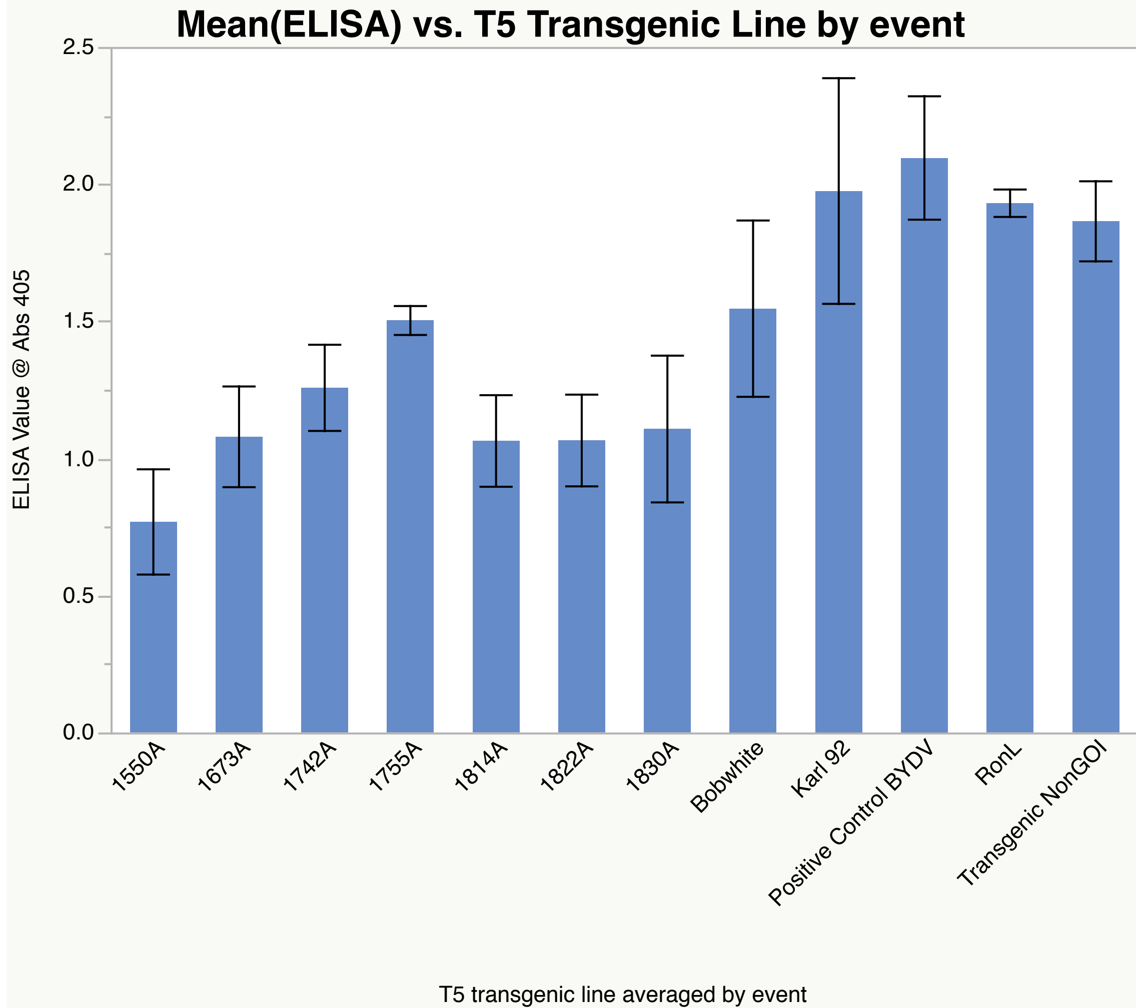


Figure 5.8: BYDV bioassay results of T_5 pooled transgenic lines showing resistance over five replicates, consisting of fifteen plants each replicate. Purified virus, 'RonL,' 'Karl 92' and transgenic non-GOI plants were used as controls. ELISA values were averaged by event. These event families were evaluated as averages, however, many individual plants from each event family had high levels of resistance when compared to controls using Dunnet's Adjustment ($p<0.01$).

Infection with SbWMV: T_3 Generation derived by T_2 bulk			
T_3 eIF4(iso)4E-2	**Total Plants**	**GOI + and Expressing**	**Resistant to SbWMV**
Event 1550A	75	32	26
Event 1822A	70	28	24
Event 1814A	73	30	24
Transgenic Bobwhite w/o GOI	17	0	0
Ernie	20	0	0
Fuller	15	0	15
Infection with SbWMV: T_3 Generation derived by T_2 bulk			
T_3 eIF4G	**Total Plants**	**GOI + and Expressing**	**Resistant to SbWMV**
Event 1673A	10	6	6
Event 1742A	29	19	15
Event 1755A	20	12	9
Event 1830A	12	7	7
Transgenic Bobwhite w/o GOI	11	0	0
Ernie	19	0	0
Fuller	18	0	18

Table 5.3: SbWMV bioassay results of T_3 pooled transgenic lines derived by T_2 bulk showing resistance based on phenotypic scores and ELISA values. 'Fuller' serves as a resistant control, while 'Ernie' serves as a susceptible control. These lines were derived from a bulk of T_2 seed, resulting in individual plants that are null-segregants as shown in the column GOI+ and expressing. Furthermore, with an absence of selection at the T_2 generation, there is variability in the levels of expression of the two transgenes.

Chapter Six - A standardized rating system for *Wheat streak mosaic virus*

Abstract

Wheat streak mosaic virus (WSMV) is one of the most prevalent viral diseases affecting wheat (*Triticum aestivum* L.) and is found in all wheat growing areas of the world. Cultivars display a variety of symptoms and severity and because there is no standardized rating system for WSMV, comparisons between studies are very difficult. Some varieties present classic symptoms of a yellow-green mosaic present on the leaves, while other varieties present with more leaf streaking. Infected wheat plants are stunted, display prostrate growth and often present with leaf curling due to mite feeding. Infected commercially grown cultivars range from extremely susceptible to resistant. However, there is little to no genetic resistance available for WSMV, making a standardized scale especially important. The proposed rating scale enables the determination and comparison of resistance to WSMV presented by a broad spectrum of cultivated varieties. The rating scale is comprised of ordinal values '1' through '9,' with '1' considered resistant, while '9' is considered very susceptible. The scale can easily be converted to from other types of rating scales, and can further separate varieties on a continuum of resistance. Some varieties considered 'moderately resistant' are in fact better than others in that same group. This scale allows a number to reflect this difference. Furthermore, a standardization of the rating scale for WSMV will allow data on virus ratings to be more easily understood by producers and industry.

Introduction

Wheat streak mosaic virus (WSMV) is one of the most prevalent viral diseases affecting wheat (*Triticum aestivum* L.) in the Great Plains of the US (Byamukama *et al*., 2014). First

reported in 1922, the disease is now found in all wheat growing areas of the world including Canada, Europe, Mexico, Australia, and Asia (Hadi *et al*., 2011). WSMV is the type member of the *Tritimovirus* genus of the family *Potyviridae* (Stenger *et al*., 1998). Occasionally, low levels of seed transmission occur (Dwyer *et al*., 2007); however, WSMV is primarily spread by the eriophyid wheat curl mite (WCM) (Slykhuis, 1955). In addition to WSMV, WCM is also capable of vectoring *Triticum mosaic virus* (Seifers *et al*., 2008) and Wheat mosaic virus, formerly known as High plains virus (Seifers *et al*., 1997). WSMV also infects other cereals including barley, corn, maize, millet, sorghum, oat, triticale and can be found in many weedy species (Seifers *et* al., 1996; Sill and Connin, 1953). Yield loss ranging from 2%-5% is common in the Great Plains, however localized fields may experience 100% yield loss, making a dramatic economic impact on producers (Figure 6.1 and Figure 6.2) (Brakke *et al*., 1987, Macneil *et al*., 1996, French and Stenger, 2004).

Currently, there is no standardized rating system for WSMV, which makes comparisons between studies very difficult. Additionally, cultivars display a variety of symptoms and severity, which can further confuse the rating process. Some varieties present classic symptoms of a yellow-green mosaic present on the leaves, while other varieties present with more leaf streaking (Figure 6.3). Commonly, infected wheat plants are stunted and display prostrate growth (Figure 6.4). Leaf curling can occur due to mite activity (Sharp *et al*., 2002).

The difficulty in rating has to do with the life cycle of the virus. Mites penetrate the leaf tissue with their stylets in order to feed (Sabelis and Bruin 1996), thereby transmitting the virus into the leaf mesophyll. From there, the virus moves down to the roots, creating a systemic infection as the virus is moves up to the upper leaves (Hipper *et al*., 2013). Typically, researchers rate on percent infection, but are more likely rating on a severity scale between 1-

100%, which can be misleading. Symptom variability may be mid-range, despite a 100% infection. Meanwhile, wheat that is very susceptible may contain a lower level of viral titer, but appear at a high severity rating. Ultimately, commercially grown cultivars will be found along a continuum of resistance ranging from extremely susceptible to resistant. A standardized scale is especially important for WSMV, because little genetic resistance is currently available. This paper will advocate the adoption of a standardized rating scale. Furthermore, a basic pathway for analysis of the rated data will be explained.

There are many disease rating scales in use for WSMV among Great Plains states. Publications from Nebraska in 2014 used a rating scale from '1-9', where '1' is completely susceptible, and '9' is resistant (Regassa *et al*., 2014). Kansas and Colorado rates '1-9' as well, however a '1' rating represents highly resistant and a rating of '9' represents highly susceptible (DeWolf *et al*., 2014, Johnson *et al*., 2014). Oklahoma rates on a scale from '1-5', with '1' defined as 'excellent' and '5' as 'very poor' (Edwards *et al*., 2014,). Texas and South Dakota rate on a scale using 'susceptible', 'moderately susceptible', 'moderately resistant', and 'resistant' (Neely *et al*., 2014, Kleinjan *et al*., 2014). A standardized rating scale will eliminate confusion when comparing differences across studies and provide meaningful information wherever wheat is grown.

Materials and Methods

Two data sets were used to validate this proposed rating scale. For the first, the data from the Kansas 2013 WSMV virus ratings were used (DeWolf, 2013). This data set consisted of 87 different varieties, including susceptible and resistant checks, analyzed over two replicates. The second data set originated from the 2014 WSMV variety trials (DeWolf, 2014), which consisted of 74 different varieties, including check varieties and consisted of five replicates. All varieties

were screened by planting five seeds of each variety in 10 cm pots containing Metro Mix 360 soil medium (SunGro, Vancouver, BC). Each year seeds were planted beginning the third week of September in the greenhouse at a temperature of 20 °C, 16 h day length under high-pressure sodium lamps, and continued every two weeks to complete the replicates.

WSMV Sidney 81 was maintained in the susceptible hard winter wheat cultivar 'Tomahawk' (PI 478006). The virus was inoculated using a finger rub technique to the second leaf of two week-old seedlings. Carborundum was dusted lightly onto the second leaf and approximately 40 μl of infected plant sap (100 mg of desiccated leaf tissue in 100 μl of 0.02 M sodium phosphate buffer, pH 7.4) was placed above the carborundum. The leaf was then pinched between the thumb and forefinger and the plant sap was pulled down the length of the leaf several times. The virus was maintained by reinoculating new seedlings every four weeks. Virus presence was evaluated by enzyme linked immunosorbent assay (ELISA) and inoculation onto the wheat cultivar 'RonL' which is resistant to WSMV (Martin *et al*. 2007). WSMV infected leaf tissue was stored by placing 3–6 cm sections of leaves onto a bed of Drierite desiccant (Hammond Drierite, Xenia, OH) covered by Fisherbrand P8 Filter paper (Fisher Pittsburgh, PA), (Cat no S47573C), in a Nunc 15 x 9 x 100 mm petri dish (Nalge, Rochester, NY), and stored at -20 °C until used.

Plants were inoculated at the three-leaf stage. All five plants were inoculated with the virus. The same plants were inoculated a second time 14 days after the first inoculation using the same procedure. Fourteen days post second inoculation plants were scored using a numerical scale 1-9, with 1 being no symptoms and 9 being severe symptoms (Figure 6.5).

Tissue samples were taken at this time from the youngest leaf of each of the infected plants. A 2.54 cm of tissue was collected from mid leaf and placed into a 2 ml screw cap tube

(LabSource, Romeoville, IL). Wheat tissue was evaluated for the presence of WSMV using double antibody sandwich (DAS) ELISA assay. The tissue was macerated by placing a ceramic bead (MP Biomedical, Solon, OH) in the tube with 300 μl of general extraction buffer (Agdia, Elkhart, IN). The tubes were placed in an MP Fastprep 24 machine (MP Biomedical) at 4 m/s for 20 s and spun in a microfuge at 13,000 x g. An additional 700 μL of general extraction buffer was added to the tube. The tube was then vortexed for 15 s and microfuged for 30 s at 13,000 xg. Supernatant was applied to WSMV Pathoscreen ELISA plates (Cat no. PSA47001) and the manufacturers protocol was followed. Plates were read using a spectrophotometer (Biotek, Highland Park, Winooski, VT) at ABS_{405}. Resistance was determined by comparing ELISA values of each variety to those of both the susceptible and resistant controls. Statistical analysis was performed using SAS version 9.2 (SAS Institute, Cary, NC) and JMP®, Version *11.2* SAS Institute Inc., Cary, NC, 1989-2007.

Plants of representative scores of 2 through 9 were chosen for real-time PCR analysis. These included ClaraCL, Tomahawk, and mock inoculated Bobwhite, Bobwhite inoculated only once, and Bobwhite inoculated as described above. One inch of the youngest leaf was sampled for RNA. RNA extraction was completed using *mir*Vana miRNA extraction kit (Ambion Catalog number: AM1560) according to the manufacturers instructions.

Real-time RT-PCR was performed on the variety samples, transgenic samples, and controls. One µg of total RNA was used to complete first strand synthesis with random hexamers, followed by reverse transcription with Superscript II (Invitrogen) according to the manufacturer's recommendations. Primers for qRT-PCR were designed from the gene sequence of *Wheat streak mosaic virus NIb* (NCBI Genbank: U67937.1) and used to assess differences in the levels of viral titer between samples. iQ SYBR Green supermix for iCycler (BioRad, La

Jolla, CA) was used in all reactions. Three technical replicates for each biological replicate were performed for each reaction. Primer efficiencies were tested prior to use and those with efficiencies between 90-110% were used in experiments. The Cq value for the target was subtracted from the Cq value of the internal reference gene *Actin* (GenBank accession number AB181991) (Table 6.1).

Results

A total of 595 plants, from 89 different varieties over the course of two years were planted, inoculated and rated during the years 2013 and 2014 and used for evaluation of the rating scale. In order to examine the relationship between the visual scores and the ELISA values, averages were taken by variety of both the visual scores and the ELISA values. These were then plotted with ELISA averages by variety on the Y-axis, and average visual score by variety on the X-axis. A regression formula was found as y=-0.639568+0.351813x, with an r^2 value of 0.54763 (Figure 6.6). Visual scores and ELISA values were found to be highly correlated, with a p-value 1.0. Taken together, the proposed rating scale efficiently and effectively relates visual scores to ELISA values. A scale ranging from '1-9' can easily capture small differences between varieties that can be more accurately used to phenotype.

The same rating scales were used to rate 367 transgenic plants (Cruz *et al*., 2014) that had first been categorized as having the gene of interest (GOI) (WSMV CP hairpin) or not. Plants were categorized as GOI positive or negative, and whether they were considered resistant (YES or NO) (Figure 6.7). Levels of viral titer were measured with real-time PCR. Relative expression of viral gene WSMV *NIb* in 'Bobwhite', that which was inoculated one time, and that of the 'Bobwhite' that was inoculated twice produced ΔΔCT values in accordance with the standardized rating scale, as did the variety Mace, which contains the resistance gene, *Wsm1*,

ClaraCL, which contains resistance gene *Wsm2*, and Tomahawk, as a susceptible line.. Furthermore, this correlation between viral titer was reflected in 'Bobwhite' that was mock-inoculated, scored a '1', which was inoculated one time, rated a '6', and that of the Bobwhite inoculated two times, scored at '9'. qPCR results of the varieties tested are displayed in Figure 6.7, while the phenotypic scores of those varities are found in Table 6.2.

Discussion

A scale was proposed that enables the determination and comparison of resistance to WSMV presented by a broad spectrum of cultivated varieties. The rating scale is comprised of ordinal values '1' through '9,' with '1' considered resistant, while '9' is considered very susceptible. WSMV and other viral diseases prove harder to rate consistently due to their infection cycles (Hipper *et al*., 2013). Fungal diseases, such as leaf rust and stem rust, can be rated more easily based on their degree of foliar symtoms (Kolmer *et al*., 2013; James, 1971). Virus ratings produce a unique challenge. There is a wider variety of ELISA values associated with mid-range scores in all varieties tested in this study. This could be due to the subtle differences in visual appearance at ratings in the mid-range. Resulting scores could possibly be attributed to the efficiency of virus replication and or movement in certain varieties. However, the use of many data points allowed the means to accurately reflect the visual scoring and ELISA values. A clear relationship exists between the increase in viral titer, and phenotypic score. While viral presence could be detected in qPCR in all samples, the resistant varieties, Mace and ClaraCL containing resistance gene *Wsm1* and *Wsm2* respectively, were still considered very low, in agreement with a phenotypic score of '2.'

The standardization of rating wheat for WSMV, has additional applications for TriMV. TriMV has virtually identical symptoms to WSMV. However, together these two viruses cause an even more drastic phenotypic display (Seifers *et al*., 2008). While plants often are more dramatically stunted when co-infection is present, scoring will still produce a high rating such as '8' or '9'. This is in agreement with ELISA values and qPCR relative gene expression values as high. When viral RNA level was assayed in plants with ratings along the scale, results were consistent with the symptom severity score—the highest level of viral RNA was detected in the known susceptible lines, less in the moderately resistant varieties and very low levels of viral RNA were detected in the highly resistant varieties. However, there are certain varieties in which their phenotypic rating does not correlate with the level of viral titer. Also, the time of rating matters a great deal. Varieties such as WGRC50 and WGRC27 can appear at nearly a '9' rating two-three weeks after the first inoculation, but allowed to grow, plants appear fully recovered and will receive a '2' rating. Limitations also exist during the process of mechanical inoculation. Differences in inoculation techniques can lead to a difference in the amount of initial viral particles introduced into the plant. In this case, it is best to use a variety of known controls, and rate plants accordingly.

Plant pathologists have been using these types of scales to try to estimate disease severity for decades. Regardless of value, these types of ratings are all categorical variables. Categorical variables cannot be analyzed using ANOVA, which often make them wholly undesirable to researchers. However, there are efficient methods available to analyze this type of data. In this case, ratings on a '1-9' scale are on a clearly ordered scale, termed *ordinal variables*. Specifically, categorical variables are referred to as qualitative variables due to the fact that they are not associated with numerical values, but in this type of rating scheme, it is possible to treat

viral ratings in a quantitative manner. This can be done because these ordered scores represent an equal, incremental increase along the scale.

Applications for transgenic plants

Categorical analysis can be particularly useful for those working with transgenic wheat. In early generations, segregation of transgenes occurs (Rooke *et al*., 2003). Particle bombardment is, at this time the most commonly used method to transform wheat. Particle bombardment often leads to incorporation of high copy numbers of transgenes and their promoters, which leads to transgene silencing and possible DNA methylation in later generations (Matzke *et al*., 2000). Physical losses of transgenes also occur, as in work by Srivastava *et al*., (1996) that reported a wheat line that had physically lost the transgene in the T_3 generation. Statistical analysis of the efficacy of the transgenic plants should not be skewed by using data obtained from non-transgenic plants. Researchers often simply report that either segregation or transgene silencing has occurred, but categorical data analysis can easily be performed on the data by first categorizing the transgenic plant as having and or expressing the transgene. Using SAS, we can assign plants having the transgene as category '1', while those that do not category '2.'

Statistical approaches for analysis

Example:

```
data ELISA;
input plant score ELISA @@;
cards;
```

The proposed standard rating scale can be applied by first sorting plants into categories. Virus ratings most often have a trend association between the rating and the level of viral titer.

Most commonly, researchers use techniques for detecting viral proteins such as enzyme-linked immunosorbant assays (ELISA), reverse transcription polymerase chain reaction (RT-PCR) and real-time PCR (qPCR). Associations between phenotypic rating and other elements are also possible, such as yield loss. The first step is often to detect such a trend association. This can be accomplished using simple statistical techniques. For this example, ELISA values obtained from a previous experiment will be used. The virus ratings are designated as the variable, *X*, while the response variables, the ELISA values will be assigned as the variable, *Y*. First we must calculate, *r*, also known as the Pearson correlation, which is designed to detect the degree of linear trends.

$$r = \frac{\Sigma i, j(u_i - \bar{u})\left(v_j - \bar{v}\right)p_{ij}}{\sqrt{[\Sigma_i(u_i - \bar{u})^2 p_{i+}][\Sigma_j(v_j - \bar{v})^2 p_{+j}]}}$$

r values produced will be between -1<0<1, while an r of approximately zero will indicate linear independence. This value forms the basis of the test statistic.

This analysis is dependent on using scores that reflect the distance between the categories. This is inherent in the proposed virus rating scheme, meaning that the difference in severity between a '4' rating and a '5' rating is equal to the distance between a '7' rating and that of an '8.'

Ho: X and Y are linearly independent

Ha: X and Y are not linearly independent

The test statistic, M^2, will be produced in SAS directly.

$$M^2 = (n - 1)(r^2)$$

It is important to recognize that this test statistic requires a large sample size in order to be valid. Here, we can view *Y* as a continuous positive variable, so a normal distribution can be considered.

```
proc freq;
weight ELISA;
tables plant*score/chisq cmh1;
run;
```

There are three components of a generalized linear model (glm). The first is the random component, which identifies the response variable Y. The random component also assigns a probability distribution for Y. The second component is referred to as the systematic component. The systematic component defines the explanatory variables for the model. Finally, the link function specifies a function of the expected value (mean) of Y. This allows the glm to related the explanatory variables through a prediction equation having linear form.

In our case, our Y represents the ELISA value, which, within the range of O.D., we can consider this continuous. This way, we can assume a normal distribution. The systematic component of our model, our x_j's are our explanatory variables, which are the linear predictors. In this case, these are our phenotypic scores.

$$\alpha + \beta_1 x_1 + \cdots + \beta_k x_k$$

The mean of its probability function, the expected value of Y is μ=*E(Y)*. It is the link function that relates μ to the linear predictor. The link function, in fact links the random and the systematic components.

$$g(\mu) = \alpha + \beta_1 x_1 + \cdots + \beta_k x_k$$

This is commonly done using simple regression, where we model ELISA scores, Y, as a function of visual scores, x.

Conclusions

Temperature differences in the field may also be a source of rating discrepancies. The resistance gene, *Wsm2*, an example of a dominant single gene, breaks at temperatures exceeding approximately 24°C (Haley *et al*., 2002; Seifers *et al*., 2006; Martin *et al*., 2007; Liu *et al*., 2011). *Wsm1*, is also temperature sensitive above 24°C. *Wsm3* is not temperature sensitive to WSMV, but is to TriMV. Minor gene resistance or tolerance has been found in hexaploid bread wheat (Rahman *et al*., 1974; Martin *et al*., 1976; Seifers and Martin 1988). There are also genes for resistance to wheat curl mite, which is an alternate method to reduce the incidence of WSMV through control of the vector (Martin *et al*. 1984). These facts can complicate accurate virus ratings (Liu *et al*., 2011). It is important to be aware of these drawbacks when attempting to rate. Confusion can lead to skewed variety ratings.

This scale is can easily be converted to from other types of rating scales, and can further separate varieties on a continuum of resistance. Some varieties considered 'moderately resistant' are in fact better than others in that same group. This scale allows a number to reflect this difference. Furthermore, a standardization of the rating scale for WSMV will allow data on virus ratings to be more easily understood by producers and industry.

Figures and Tables

Figure 6.1: Wheat field decimated by WSMV, Saline Co. KS, 2011. This field of the wheat variety ‘Armour’ appears yellowed, stunted, and displaying prostrate growth. Virus presence was confirmed via ELISA. This field was ruled a complete loss by Kansas State Wheat Pathologist, Dr. Erick DeWolf.

Figure 6.2: A field with heavy WSMV infection. Plants are displaying stunting, prostrate growth, and yellow-green mosaic foliar symptoms. Saline Co., KS 2011.

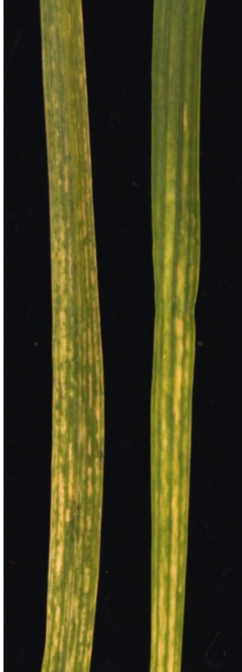


Figure 6.3: Variation in symptomology of WSMV. Left: Leaf displaying classical mosaic symptomology. Right: Leaf displaying yellow streaking

Figure 6.4: Panels 1 and 2: Wheat inoculated with WSMV displaying prostrate growth patterns exhibited by infected plants. Panel 3: Non-infected wheat plants displaying normal growth patterns.

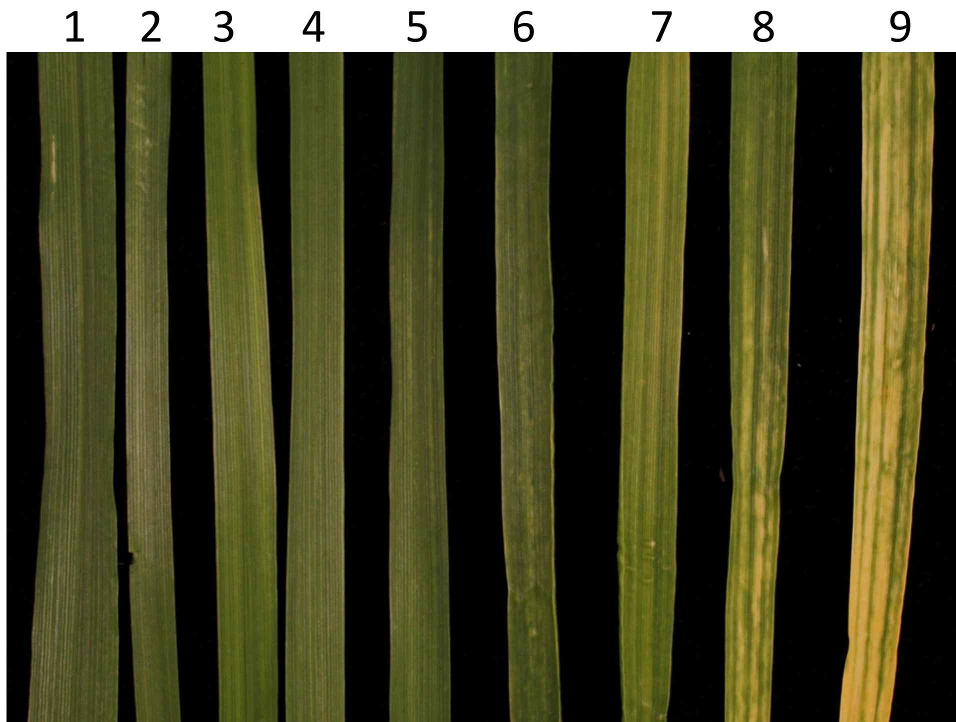


Figure 6.5: WSMV rating scale. From left to right, resistant to susceptible rating scale 1-9 This scale displays an effective continuum of the severity of phenotypic virus ratings ranging from clean leaves (1), to the beginnings of yellowing and mosaics in the leaves (2-4). At rating 5, streaks in the leaves are apparent, but cover less than 50% of the leaf. Rating 6 covers slightly more than 50% of the leaf with streaking. Ratings 7, 8, and 9 continue from full leaf mosaic patterns with an equal increase in severity at every level.

Primer Name	Sequence 5’ to 3’	Tm (°C)	Use
WSMVNIaqa	CATCCTCGTTGAAGACAATC (Sense) CTCCTCTCTGTTCCTCATAC (AntiSense)	50.9°C 51.4°C	WSMV titer detection
ACTqa	GTTCTCAGTGGAGGTTCTA (Sense) CTTTCAGGTGGTGCAATAA (AntiSense)	50.7°C 50.3°C	Housekeeping gene *Actin*

Table 6.1: Primers used in real-time PCR

Y=-0.639568+0.351813x

R^2=0.54763

Mean ELISA by variety vs. Mean Visual Score by variety

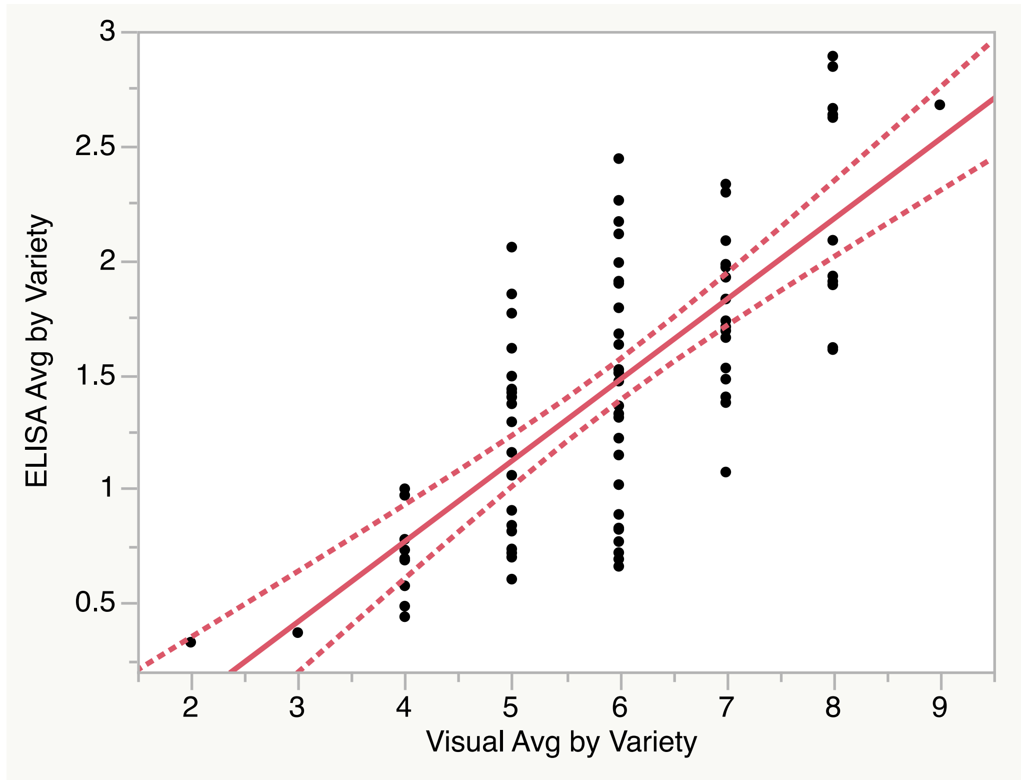


Figure 6.6: Graph of Average ELISA values by variety read at O.D. 405 vs. Visual Phenotypic Rating Average by variety. The dots represent the average of five replicates of WSMV infected samples by variety of ELISA Abs $_{405}$ vs. phenotypic score. The solid red line represents the prediction equation, while the dashed read lines represent the confidence intervals of α=0.05.

GOI By RESISTANT

Count Total % Col % Row % Expected	YES	NO	
POSITIVE	63 17.21 70.00 92.65 16.7213	5 1.37 1.81 7.35 51.2787	68 18.58
NEGATIVE	27 7.38 30.00 9.06 73.2787	271 74.04 98.19 90.94 224.721	298 81.42
	90 24.59	276 75.41	366

Figure 6.7: Categorizing transgenic plants: Categorizing GOI Negative and Positive Plants by resistant. The first row represents the number count, the second row represents the percent of the total, the third row represents the percent by column, row four represents the percent by row, and the fifth row represents the expected value.

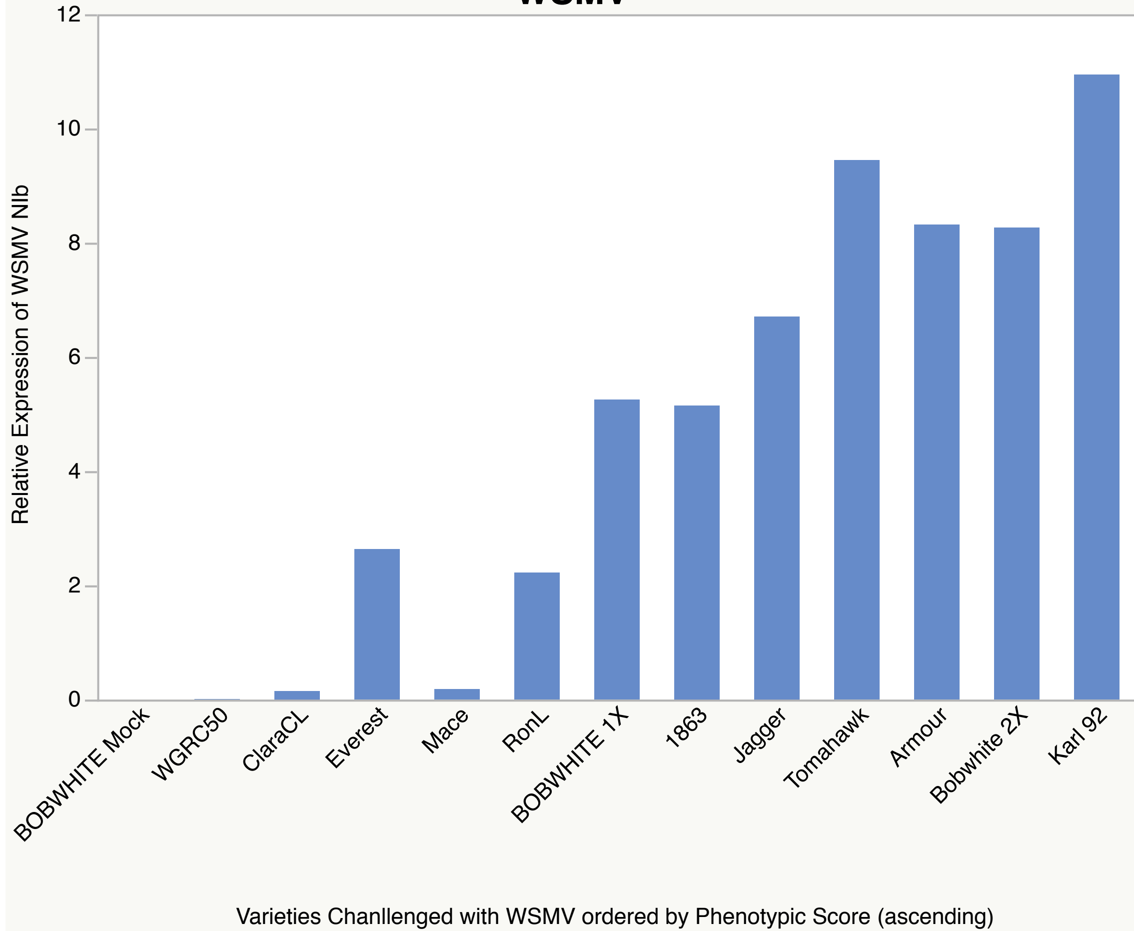


Figure 6.8: Real time analysis of the relative expression of WSMV NIb gene to measure the levels of viral titer in varieties.

Variety	Phenotypic Rating
Mock-Inoculated Bobwhite	1
Bobwhite 1X	4
Bobwhite 2X	9
Clara CL	2
1863	5
Karl 92	9
Mace	2
Tomahawk	8
WGRC 50	1
RonL	2
Everest	4
Armour	9

Table 6.2: Phenotypic scores of plants displayed in Figure 6.8 rated at two week after the second inoculation with WSMV.

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