RESISTANCE TO *WHEAT STREAK MOSAIC VIRUS* AND *TRITICUM* MOSAIC VIRUS IN WHEAT MEDIATED BY RNAI

by

LUISA FERNANDA CRUZ

B.S., National University of Colombia, 2006

A THESIS

submitted in partial fulfillment of the requirements for the degree

MASTER OF SCIENCE

Department of Plant Pathology
College of Agriculture

KANSAS STATE UNIVERSITY
Manhattan, Kansas

2009

Approved by:

Co-Major Professor
Harold Trick

Approved by:

Co-Major Professor
John Fellers

Abstract

Wheat streak mosaic virus (WSMV) and Triticum mosaic virus (TriMV), are two of the major viruses in the Great Plains of the United States. Cultural practices and mite vector control are the primary methods of disease management; however, they are not fully effective. Resistant varieties are also deployed, although some of the lines present temperature sensitive resistance or negative agronomic properties are linked to resistance. Alternative approaches to viral resistance are needed. 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. RNAi reduces the expression of specific genes by targeting the accumulation of mRNA. The mechanism is activated by the presence of dsRNA, which is processed into small non-coding sequence that serves as a guide for degrading RNA in a sequence specific manner. In this work, the RNAi approach was used to elicit resistance against WSMV and TriMV. Immature embryos of the wheat cv 'Bobwhite' were independently co-transformed with pAHC20, containing the bar gene for glufosinate selection, and either the hairpin construct targeting the coat protein of WSMV or TriMV. After tissue culture, PCR was used to determined the presence of the RNAi CP transgene in putative transformed plants. Eight WSMV and ten TriMV CP RNAi transgenic plants were obtained from the bombardment experiments. Transgenic T_1 and T_2 seeds were collected and transgene expression was established through RT-PCR. In order to determine viral resistance, T_1 and T_2 progeny was mechanically inoculated. ELISA results indicated a differential resistance response among the tillers evaluated in each line in T_1 generation for both WSMV and TriMV constructs. In T_1 generation resistance was seen in up to 60% of the plants evaluated for both constructs, although some events that showed transgene presence did not exhibited resistant phenotype. Analyses of transgene presence and expression in T_2 generation evidenced events of transgene silencing and deletion. Regardless of these phenomena, consistent resistance response in two lines of WSMV CP construct and one TriMV CP transgenic line was found.

Table of Contents

List of Figures iv
List of Tables v
Acknowledgements vi
Introduction 1
Materials and Methods 12
Plasmid Construction: 12
Plant Transformation 14
Plant material 14
Bombardment 15
Tissue culture 15
Plant Evaluation 16
Liberty painting 16
PCR gene detection 16
Southern Hybridization 17
T_1 and T_2 Generation Evaluation 18
Bioassays 19
ELISA T_1 and T_2 generation 19
siRNA Detection 20
Small RNA fraction and RNA hybridization 20
Segregation analyses 21
Data Analysis 21
Results 22
Expression of WSMV CP RNAi vector: 22
Expression of TriMV CP RNAi vector: 25
Other Constructs from WSMV: 29
Discussion 31
Conclusion and future work 36
References And/Or Bibliography 63

List of Figures

Figure 1. Schematic representation RNAi vector construction. 50
Figure 2. Tissue culture selection cycles in dark. 51
Figure 3. Tissue culture regeneration cycles 52
Figure 4. Schematic diagram of pAHC20 53
Figure 5. Wheat leaves painted with 0.2% Lyberty herbicide.....54
Figure 6. Phenotypes observed in transgenic and non-transgenic. 55
Figure 7. PCR analyses on gDNA of T_0 WSMV CP RNAi transgenic wheat.. 56
Figure 8. Molecular analyses of the T_2 WSMV CP RNAi transgenic plant line 110-B8. 57
Figure 9. Molecular analyses of the T2 WSMV CP RNAi line 147- a1.....58
Figure 10. PCR analyses on gDNA of T_0 TriMV CP RNAi transgenic wheat. M, 100 bp DNA 59
Figure 11. Molecualar analyses of the T2 TriMV CP RNAi transgenic plants line 201- A19. 60
Figure 12. ELISA results T_1 and T_2 generation WSMV CP hairpin construct.. 61
Figure 13. ELISA results T_1 and T_2 generation TriMV CP hairpin construct.. 62

List of Tables

Table 1. Wheat tissue culture media used in these studies. 38
Table 2. Primer sequence and descriptions of sequences used in these studies. 39
Table 3. Summary of T_0 transgenic plants analyses. 41
Table 4. PCR and RT-PCR analyses T_1 CP RNAi WSMV plants. 41
Table 5. WSMV detection by RT-PCR of T_1 progeny used in preliminary bioassay. 42
Table 6. PCR and RT-PCR analyses of T_1 generation WSMV CP RNAi plants. 42
Table 7. Correlations of ELISA results and molecular analyses of T_1 WSMV CP RNAi plants. . 43
Table 8. PCR and RT-PCR of T_2WSMV CP RNAi progeny. 43
Table 9. Correlations of ELISA results & molecular analyses of T_2 WSMV CP RNAi progeny. 44
Table 10. Molecular analyses of T_1 TriMV CP RNAi plants. 44
Table 11. Correlations of ELISA results and molecular analyses of T_1 TriMV RNAi plants. 45
Table 12. Molecular analysis of T_2 TriMV RNAi CP progeny. 45
Table 13. Correlations of ELISA results and molecular analyses of T_2 TriMV CP RNAi plants. 45
Table 14. Segregation analyses for T1 generation WSMV and TriMV hairpin construct. 46
Table 15. Contingency table transgene presence and virus presence, WSMV CP RNAi T_1. 47
Table 16. Contingency table transgene presence and virus presence, WSMV CP RNAi T_2. 47
Table 17. Contingency between transgene presence and virus presence, TriMV CP RNAi T_1 ... 47
Table 18. Contingency table transgene presence and virus presence, TriMV CP RNAi T_2. 48
Table 19. Contingency table transgene expression and virus presence, WSMV CP RNAi T_1. 48
Table 20. Contingency table transgene expression and virus presence, WSMV CP RNAi T_2 48
Table 21. Contingency table transgene expression and virus presence, TriMV CP RNAi T1 49

Acknowledgements

I would like to thank Dr. Harold Trick for providing me the opportunity to be part of his lab and to study at Kansas State University. I am grateful for his guidance during these years. I also would like to thank Dr. John Fellers for his support and assistance and Dr. Anna Whitfield for being part of my committee. I am also grateful for all the support of the members of the lab have given me. Julie Essig for all the knowledge and advice, Sheila Stevens for all her assistance in the laboratory techniques, and Dr. Jiarui Li and Dr. Jung Hoon Lee for answering all my questions and assistance with the experiments. I also would like to thank Hyeonju Lee for all her help. I am also thankful with the Plant Pathology Department graduate students and friends for all the encouragement and support. My most sincerely gratitude goes to my family here with me in the United States, David, Juan and Carolina and to my parents in Colombia.

Introduction

The United States ranks third in the world for annual wheat production. According to the USDA Economic Research Service, in 2008 63.15 million acres were planted and the overall production was 2,499.52 million bushels. With half of the U.S. wheat production being exported, the U.S. produces about 13% of the world's wheat and supplies about 25% of the world's wheat export market. Over 70% of the wheat is used for food products, approximately 22% is used for animal feed and residuals, and the remainder is used for seed (US Environmental Protection Agency, epa.gov/oecaagct/ag101/cropmajor.html). Over 240,000 farms in the United States produce wheat and although wheat is grown in most of the 50 states, approximately two-thirds of total U.S. wheat production originates from the Great Plains. Kansas leads the nation in wheat production and is responsible for nearly 20 percent of total domestic production. In 2008, 9.6 million acres of wheat were planted in Kansas, which produced 367 million bushels with a market value of near $2 billion (Kansas Wheat Commission, www.kswheat.com).

A wide range of pathogens attack wheat and collectively are responsible for approximately 13 to 15% of the annual crop losses. *Agropyron mosaic virus, Wheat soil borne mosaic virus, Wheat spindle streak mosaic virus, Wheat American striate virus, Barley yellow dwarf mosaic virus, High Plains virus* (HPV), *Wheat streak mosaic virus* (WSMV) and *Triticum* mosaic virus (TriMV) (Siefers et al 2008) are the most economically important viruses. The annual losses caused by WSMV are estimated to be 2.5% (Bockus et al., 2001), but can account for over one-half of the disease loss. In 2006, Kansas growers suffered an estimated $109 million in losses due to WSMV alone (Kansas Wheat Commission, virus-resistant germplasm). The interaction of WSMV with other viruses, including HPV and TriMV, can increase the severity of symptoms and yield losses (Kansas State University research and extension, www.oznet.ksu.edu/library/plant2/ep145.pdf).

WSMV is a member of the family *Potyviridae* genus Tritimovirus (Stenger et al., 1998) and was first discovered in Nebraska in 1922. Virion particles are flexuous, non-enveloped and filamentous in shape, 690 to 700 nm long, and are made up of approximately 2000 copies the coat protein (CP) encapsulating the genetic material. The WSMV genome consists of a positive-

sense, single stranded RNA of 9,384 nt in length that encodes a single open reading frame of 3,035 amino acids (Stenger et al., 1998). The RNA genome has a 5'- viral protein genome linked VPg and carries a 3'- polyadenylated tail (Fauquet et al., 2005). Potyviruses express the genome as a single polyprotein and use a post-translational proteolytic processing strategy utilizing three encoded proteinases to produce functional proteins (Hull, 2002). The proteinases are: P1, a trypsin-like serine proteinase at the amino- terminal end of the polyprotein and has *cis* auto catalytic activity; nuclear inclusion protein A (NIa), the major proteinase of potyviruses acting in *cis* and *trans*; and helper component (HC-Pro), a conserved carboxy-terminal cysteine proteinase domain that acts in *cis* to cleave the HC-Pro/P3 junction of the viral polyprotein (Stenger et al., 2006; Urcuqui-Inchima et al., 2001). The polyprotein cleavage results in seven smaller proteins denoted as P1, HC-Pro, P3, cylindrical inclusion (CI) protein, NIa, nuclear inclusion B (NIb), CP, and two small proteins known as 6K1 and 6K2 (Riechmann, et al., 1992).

The majority of *Potyviridae* are aphid transmitted, however Tritimoviruses are mite-transmitted. WSMV and TriMV are transmitted semi-persistently by the wheat curl mite *Aceria tosichella* Keifer (Slykhuis, 1955; Siefers et al., 2009). The multifunctional HC-Pro has been implicated in different steps of the potyvirus life cycle (Maia et al., 1996) in addition to its function in virus-vector interaction (Stenger et al., 2005). Three HC-Pro regions have been described. The amino terminus is implicated in vector transmission (Blanc et al., 1998), the carboxy-terminus is a proteinase domain involved in polyprotein maturation (Maia et al., 1996), and the protein central region has an essential role in viral amplification and systemic movement (Croin et al., 1995). However, in 2005, Stenger and coworkers studied WSMV HC-Pro and they confirmed that mutations in WSMV HC-Pro abolish long-distance movement. Their experiments also indicate that HC-Pro was not required for replication, cell-to-cell movement, or encapsidation of WSMV, although it might be involved in optimization some of these processes. HC-Pro is implicated in silencing suppression (Anandalakshmi et al., 1998) and is thought to inhibit the accumulation of siRNA by binding the 21nt short class siRNA and by interfering with the methylation of viral RNAs or preventing RISC assembly (Mérai et al., 2006).

The CP is involved in aphid transmission and has been divided in three domains. The C-terminal and the N-terminal expose on the surface of the particle and implicated in systemic movement, and the conserved central domain implicated in cell-to-cell and virus encapsidation

(Dolja et al., 1994). The DAG motif, located in the N-terminal domain, is conserved among the aphid-transmissible Potyviruses and required in the interaction of HC-Pro and CP (Blanc et al., 1997). WSMV CP lacks this motif and an interaction with the HC-Pro has not been found, suggesting a different mechanism for virus transmission in this genus of virus (Stenger et al., 2005).

Other proteins, such as NIa, are involved in genome amplification. In addition to its activity in protein cleavage, the N-terminal VPg domain functions in viral replication and host specificity. VPg is covalently attached to the viral RNA and interacts with the host cap binding protein eukaryotic translation initiation factor 4E (eIF4E; Leonard et al., 2000). This interaction is required for infection and could also lead the initiation of translation of viral genome (Thivierge et. al., 2005). NIa interacts with NIb (the potyviral RNA–dependent RNA polymerase, RdRp). These proteins are co-localized in the inclusion bodies at the nucleolus and cytoplasm of infected cells. NIb replicase activity is found in the cytoplasm and is associated with the replication complex during viral genome amplification (Fellers et al., 1998). Another protein linked with replication is the 6k2 peptide. It is associated with the vesicular compartment derived from the endoplasmic reticulum. The binding occurs via its central hydrophobic domain and it is postulated that 6k2 anchors the viral replication complex to ER-like membranes (Schaad et al., 1997).

The cylindrical inclusion protein (CI) belongs the 'super family 2' of RNA helicases characterized for the presence of seven conserved segments. Segment I is important for NTP and RNA binding activity, segment Ia has unknown function, segment II is involved in NTPase activity, segment III and IV are highly conserved, segment V appears to be involved in ATPase activity required for RNA unwinding (Fernandez et al., 1995), and segment VI has been shown to have RNA binding activity (Fernandez and Garcia, 1996) . CI protein has been implicated in virus cell-to-cell movement. CI localized close to the plasmodesmata during early stages of infection where ATPase activity has been found. ATPase and helicase activity are probably required for cell-to-cell viral transport, thus CI may be involved in this function (Carrington et al., 1998). CI also has been implicated in replication but this function is still unknown (Urcuqui-Ichima et al., 2001).

Triticum mosaic virus (TriMV) is a new virus isolated in Kansas in 2006 (Siefers et al., 2008). TriMV was discovered infecting a WSMV-resistant wheat cultivar RonL, and symptoms are indiscernible from those caused by WSMV. Serological analyses by ELISA of wheat extracts gave negative reactions against antibodies specific to WSMV, other potyviruses and *High plains virus*. Electron microscopy of cesium chloride purified virus preparations identified virus like particles that were flexuous and rod shaped and 15nm wide 800nm in length. Sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) analyses of these preparations evidenced a 35 KDa protein encoded by 882 nt corresponding to the coat protein of the infectious agent. Amino acid sequence of this protein exhibited 49% similarity to *Sugarcane streak mosaic virus* (SCSMV), a member of the family *Potyviridae*. To determine the vector of TriMV, Seifers and co-workers screened two possible candidate vectors, the bird cherry oat aphid (*Rhopalosiphum padi* L.) and the wheat curl mite (*Aceria tosichella* Keifer), both present in the geographical localization where TriMV was found. The results showed that wheat curl mite is the vector of TriMV and virus transmission can occur individually (WSMV or TriMV alone) or together (Seifers et al., 2009).

Primary control strategies for WSMV and TriMV are the same. They are focused on limiting the increase and spread of vector population, as well as minimizing the source of inoculum. One of the main recommendations for control is the eradication of alternative mite and WSMV hosts such as volunteer wheat and grassy weeds that can act as a virus reservoir during the non-crop season. Planting dates are also important in order to decrease the disease impact. In the case of winter wheat, planting earlier than the recommended date allows mite establishment and reproduction (Kansas State University, Extension Plant pathology, www.oznet.ksu.edu/path-ext/factSheets). Another point to take into consideration is related to the cultivar susceptibility and genetic resistance. For WSMV, 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. Other sources of unknown origin are present in the germplasm line CO960293-2 (Haley et al., 2002) and in the cultivar RonL (Seifers et al., 2007). These resistance sources are temperature sensitive and not effective at temperatures above 18°C. (Seifers et al., 2006, 2007). Friebe and co-workers (2009) reported TriMV genetic resistance, in wheat containing also the *Wsm1* gene. In this study, the

Th. intermedium translocation T4DL·4JsS segment was shortened in order to reduce the detrimental effects affecting bread-making quality and other agronomic parameters. In their experiments were recovered one proximal (rec36) and four distal (rec45, rec64, rec87, rec213) primary recombinants. The recombinants together with resistant and susceptible controls were screened based on phenotypic symptoms and ELISA for the resistance to TriMV and WSMV. Their findings indicate that distal recombinants rec45, rec64, rec87, and rec213 were resistant to both WSMV and TriMV at low temperatures of 18°C and susceptible at 24°C (Friebe et al., 2009).

Mechanisms of plant viral defense

R-mediated resistance to viruses

Most of the identified resistance genes that confer resistance against virus belong to the nucleotide binding site-leucine rich repeats (NBS-LRR) family. Examples are the *N* gene, from *Nicotiana sp*. conferring resistance to *Tobacco mosaic virus: Rx1* and *Rx2,* cloned from *Solanum tuberosum,* conferring resistance to *Potato virus X*; *Sw-5,* cloned from *Lycopersicon sp.* which confers resistance to *Tomato spotted wilt virus; HRT,* cloned from *Arabidopsis thaliana* ecotype Dijon-17 and *RCY1* cloned from *A. thaliana* ecotype C24, which confers resistance to *Cucumber mosaic virus* strain Y. Members of this family are subsequently divided in two subgroups depending on the N-terminal domain which can contain either a coiled-coil (CC) or Toll-interleukin-1 receptor (TIR). Corresponding with intracellular nature of viruses, NBS-LRR products do not contain a transmembrane domain (Maule et al., 2007). Most R genes operate through gene-for-gene recognition of the pathogen, where each R gene confers resistance to a specific pathogen avr gene (Flor, 1971). The specificity is believed to be conferred by the leucine rich repeats (Farnham and Baulcombe, 2006).

Hypersensitive response is typically associated with gene-for-gene resistance in which the plant prevents the spread of the infection by inducing programmed cell death and confining the pathogen to the lesions (Soosar et al., 2005). However, NBS-LRR virus resistance genes frequently lead to complete resistance not associated with cell death (Maule et al., 2007). Different viral components have been characterized as avr determinants, such as the CP, movement proteins, replicases, as well as the potyviral protein genome-linked VPg (Maule et al., 2007). Two models have been proposed, to explain the interaction between R genes and their

cognate avr determinants. The first hypothesis proposes the direct interaction between receptor (R gene) and ligand (avr). A second hypothesis called the 'guard hypothesis' suggests an additional association of R proteins with host proteins that act as negative regulators. When infection occurs, the pathogen causes modifications in the structure of the host proteins. The R gene detects these changes and is activated; the signaling cascade is initiated, resulting in resistance response (Dangl and Jones, 2001).

Viruses depend on host factors to complete their infection cycle. The eukaryotic initiation factor complex has been shown to play an important role in cell-to-cell movement, translation, and replication of diverse families of viruses. In many cases, these factors are considered pathogenicity determinants. The interaction of the potyviral VPg and the eukaryotic translation initiation factors eIF4E and eIF4G have been confirmed as essential for infection (Diaz- Pendon et al, 2004). Mutant alleles of the eukaryotic translation initiation factors can confer recessive resistance to specific virus as demonstrated in mutagenic analyses in Arabidopsis where the susceptibility to *Clover yellow vein virus* (ClYVV) and *Turnip mosaic virus* (TuMV) was tested. The experiment indicated that ClYVV accumulates in leaves of mutant plants lacking eIF(iso)4E, but not in mutant plants lacking eIF4E. In contrast, TuMV multiplied in mutant plants lacking eIF4E, but not in mutant plants lacking eIF(iso)4E. These experiments suggest the selective requirement of some of the elements of the eukaryotic translation initiation complex in potyviral infection (Sato et al., 2005). Additional evidence also suggests the requirement of eIF4G for the interaction eIF4F/VPg or its direct requirement for infection. Arabidopsis mutants knocked-out for eIF4G genes demonstrated the selective recruitment of the eIF4G isoforms for the infection of ClYVV, TuMV, *Plum pox virus* (PPV), and *Lettuce mosaic virus* (LMV) (Nicaisea et al., 2007). In barley, two genes, *rym4* and the allelic *rym5*, located in the telomeric region of chromosome 3HL, were found to confer recessive resistance to different strains of the *Barley yellow mosaic virus* (BaYMV) and *Barley mild mosaic virus* (BaMMV). The barley translation initiation factor E (eiF4E) was mapped to the same chromosomal interval and the gene product contains unique amino acid substitutions compared with the wild-type protein. The polymorphic residues were identified at the mRNA cap-binding pocket. (Kanyuka et al, 2005; Stein et al., 2005). Stable transformation of resistance genotypes with the full-length cDNA of the eIF4E from susceptible plants induced susceptibility to BaMMV (Stein et al., 2005).

Posttranscriptional gene silencing

RNAi discovery history began in 1990 when Richard Jorgensen and colleagues attempted to over express chalcone synthase (CHS), an enzyme involved in production of the anthocyanin pigments, by introducing a chimeric petunia CHS gene with the goal to increase the purple color in petunias. Unexpectedly, the transgenic plants produced variegate flowers or even white. They called this phenomenon "co-suppression" (Napoli *et al*., 1990). In fungal systems, the phenomena was called "quelling" and was observed in *Neurospora crassa* during efforts to increase the production of an orange pigment by introducing extra copies of *al1,* a gene involved in the production of carotenoid pigments. Some of the obtained transformants showed albino phenotype (Baulcombe, 2004).

Andrew Fire and Craig Mello (1998) studied the effect of injected RNA into *Caenorhabditis elegans.* The researchers used RNA from *unc22,* a gene that encodes for a non-essential myofilament protein. The reduction of this protein causes twisted phenotype. In their experiment they tested purified single sense and antisense strands, and double strand mixtures. They found that the double strand caused the strongest reduction of the protein and twitching phenotype. The same phenotype was exhibited by the progeny of the injected worms, which indicated the heritability of the silencing mechanism, additionally they noticed the requirement of few dsRNA molecules to accomplish full silencing and its specificity targeting the mRNA corresponding to the introduced one (Fire et al., 1998). Investigations in diverse organisms have shown common features and conserved elements. The mechanism of action of gene silencing is similar for different taxonomic groups among the eukaryotic organisms. RNA silencing was recognized as an antiviral regulator that protects organisms from RNA viruses' infection, preventing the random integration of transposable elements (Mello and Conte, 2004). RNA interference (RNAi) and related RNA silencing mechanisms have been also recognized for regulation of gene expression at both the transcriptional and posttranscriptional level (Buker et al., 2007).

The critical common component of posttranscriptional gene silencing is an RNase III–like enzyme (Dicer) showing specificity for double-stranded RNA and was initially identified in *Drosophila* extracts (Bernstein et al., 2001). Dicers contain four distinct domains: a helicase domain, dual RNAase III motifs, a dsRNA binding domain and a PAZ domain. This enzyme is

involved in the initiation of RNAi by processing the dsRNA into small interfering RNA (siRNA), 21-27 nucleotides long with 5'-phosphate and 3'-hydroxyl termini, and two to three nucleotide 3' overhang (Andrew and Baulcombe, 1999). siRNA serves as a specific guide for the RNA-induced silencing complex (RISC) to recognize target messenger RNAs by homologous base pairing and directs cleavage of the target mRNAs (Hammond et al. 2002). The main components of the RISC belong to the family of Argonaute proteins that contain two typical domains; a conserved PAZ domain that has an RNA- binding factor and specifically recognizes the 3'overhang of double stranded siRNA (Yan et al., 2003) and a PIWI domain that contain 'slicer' catalytic activity being able to cleave RNA in RNA/siRNA complex (Hall, 2005). The signal of RNA silencing produces a systemic effect where the host RNA-dependent-RNA polymerase plays an important role using siRNA as a primer further generating a dsRNA substrate for Dicer. The signal is transmitted through the plasmodesmata and phloem (Jorgensen, 2002). A mobile silencing signal could move either with or ahead of the virus to silence the viral RNA before, or at the same time (Baulcombe, 2004).

In plants, the post transcriptional silencing mechanism acts as a natural defense strategy for virus infections. RNA silencing allows cells to distinguish non-self mRNA, which later will be targeted for degradation (Pooggin et al. 2001). RNAi process involves three basic features; induction by dsRNA, processing of dsRNA into 21-25nt siRNA, and finally the inhibitory action of the effector complex that targets mRNA homologous to siRNA previously incorporated (Ruiz & Olivie, 2007). DNA and RNA viruses, as well as transgenes that contain hairpin structures, can induce RNA silencing. In plant viral infection, viruses with secondary-structure, a feature of single-stranded viral RNA, might be the trigger for RNA silencing (Soosaar et al., 2005).

Pathogen derived resistance is a concept based on the use of pathogen genes and their expression in a host. This generates a product that interferes with the pathogenic process by acting as a negative regulator and conferring disease resistance to the host (Standford et al., 1985). Beachy et al. in 1986 published the first study probing this theory in plant virus. Transgenic plants expressing the CP of *Tobacco mosaic virus* (TMV) were challenged with TMV. The authors found that the plants displayed no symptoms or had a delay in the appearance of symptoms (Beachy et al. 1990). John Lindbo and William Dougherty (1993) expressed the CP of *Tobacco etch virus* (TEV) in a series of experiments using translational and non-

translatable version of CP. Their findings were opposite to the expected. Several lines expressing the non-translatable version of CP showed complete resistance to TEV. In contrast, the lines corresponding to the CP translatable version showed systemic recovery phenotype in the new leaves. Resistant lines were susceptible to the closely related virus *Potato virus Y*, confirming the specificity of this mechanism. Analyses of transgene transcription level and steady-state level of TEV-RNA in both TEV-recovery and unchallenged transgenic plants, showed a similar transgene transcription rates but a notable reduction in steady-stage level in recovered plants. These results indicated that the mechanism involved in the resistance response was mediated in a sequence specific manner at the cytoplasmatic level (Linbdo and Dougherty, 1993). In 1998, Tanzer and co-workers characterized the post-transcriptional suppression in the TEV CP transgenic lines. The authors investigated characteristics of two sets of low molecular weight RNAs that appear just in silenced tissue, and determined a correlation between the decrease of full length of transgenic mRNA and the increase in the levels of low molecular weight RNAs. Their hypothesis established that transgene and viral RNA silencing share a common mechanism, which occurs in the cytoplasm and does not require ongoing translation (Tanzer et al., 1997).

The development and implementation of methods to enhance the natural plant defense response include artificial introduction of single stranded RNA, dsRNA or miRNA constructs (Meister and Tuschl, 2004). Some authors propose that single sense transgene, induced-RNA silencing requires the plant-encoded RNA-dependent RNA polymerase to produce a complementary strand (Beclin et al., 2002). This hypothesis would explain higher efficiency found with inverted repeats transgenes that are directly processed into siRNA. (Helliwell & Waterhouse, 2005).

Hairpin efficiency was initially exploited by Smith and coworkers (2000) in *Nicotiana tabacum* plants transformed with a construct encoding a single self-complementary hairpin RNA containing 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. Their results indicate 65% of the plants were immune. Next, 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. Separate sense and antisense

constructs where transformed into the tobacco plants and showed just 7% and 4 % of immune plants, respectively (Smith et al. 2000). Bucher and colleagues (2006) report multiple viral resistances by using a single transgene construct. 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 construct was obtained from 150 bp fragments of the N gene of each virus. The sequence segments where fused by PCR and cloned to form inverted repeats, which flanked an intron obtained from *Arabidopsis thaliana*. The authors determined that virus resistance frequencies of over 80% could be obtained to the four different tospoviruses in the same mixed infection. The resistance was correlated with siRNA presence (Bucher et al., 2006).

Micro RNAs, miRNAs, are known to be important regulators of plant development and have been implemented to confer virus resistance by adapting their original sequence to target specific viral transcripts. Niu and colleagues (2006) used a 273 bp miRNA precursor (hairpin structure), miR159, from *A. thaliana* as backbone to express an artificial miRNA, targeting two viral silencing suppressors, p69 of *Turnip yellow mosaic virus* (TYMV) and HC-Pro of *Turnip mosaic virus* (TuMV). The artificial miRNA (amiRNA) were transcribed independently for each virus in addition to a dimeric pre-miRNA containing both. Their findings indicate that, transgenic plants expressing the miRNA displayed normal morphology and 100% virus resistance in single or mixed infections depending on the miRNA. Additionally the resistance was maintained at 15ºC, temperature that typically compromises siRNA gene silencing (Niu et al., 2006).

Virus-induced gene silencing systems (VIGS) are used as tool for the analysis of gene function in plants. In VIGS, viruses are engineered to carry sequences derived from plant genome transcripts, activating the host sequence-specific RNA degradation system. This mechanism targets the plant sequence RNA encoded in the viral genome for degradation. This technique also has been used for knocking down genes and screening for desirable traits such as disease resistance or drought tolerance (Gura, 2000**).**

WSMV transgenic resistance

A previous study on WSMV pathogen derived resistance using the full length of potyviral replicase NIb found a substantial delay in symptoms development or asymptomatic

plants. The transgene expression and viral resistance were evaluated in lines of the T_2 and T_3 generations of NIb transgenic material. The phenotypical observations made from the transgenic material at three different time points indicate various degrees of resistance response including recovery phenotype, new leaves that exhibit no symptoms and were negative in ELISA detection, which. Interestingly five out of six lines evaluated did not show transgene mRNA expression (Sivamani et al., 2000).

Independent experiments have been done using the CP of WSMV. Sivamani and co-workers transformed wheat with a construct containing the full length of the WSMV coat protein. One out of five lines that were evaluated showed resistance to WSMV, the observed resistance corresponded to recovery phenotype. Oppose to the previous results, the resistant line showed high levels of CP mRNA expression but not CP protein expression. Another remark was the high number of transgene copies in this line (Sivanami et al., 2002). A second work using the full length of the CP of WSMV was made by Li and colleagues. As was found in previous studies, the authors found strong resistance in some of the lines containing the transgene evaluated for the first generation of plants, however analyses of T_2 and T_3 transgene loss or silencing and the corresponding susceptible phenotypes (Li et al., 2005).

The objectives of my study were: 1. Designed RNAi hairpin constructs from sequence the CP of WSMV and TriMV; 2. Produce transgenic wheat via biolistic approach; 3. Characterize transgenic plants at molecular level; 4. Perform bioassays in T_1 and T_2 progeny of transgenic material and; 5. Detect the siRNA molecules in resistant lines.

Materials and Methods

Plasmid Construction:

Primers were designed from the sequence of the coat protein (CP) of *Triticum* mosaic virus (TriMV) (GenBank Accession #EF173696) and *Wheat streak mosaic virus* (WSMV) (GenBank Accession #AF057533) using Integrated DNA Technologies, Primer Quest and OligoAnalyzer 3.0 program (www.idtdna.com). For TriMV CP gene amplification, the primer sequences of 5'-CACCGATAGACGATGCGACTGGGCAAAT-3' and 5'-TCTGTTCCTGTGGTGAAAGCTGGT -3'were selected for the forward and reverse primers, respectively. For WSMV CP gene amplification, the primer sequences of 5'-CACCAATGCAGGCAAGGACAATGAGCAG-3'and 5'-TCTGTTCCTGTGGTGAAAGCTGGT-3' were selected. The sequence of CACC was added to the 5' end of both forward primers to allow the directional cloning of the PCR fragment into the entry vector pENTER-D/TOPO (Invitrogen, Carlsbad, CA).

RNA was extracted using TRIZOL® (Invitrogen, Carlsbad, CA) isolation, according to the manufacturer's instructions. One hundred mg of symptomatic leaf tissue was ground in liquid nitrogen, homogenized in 1ml of TRIZOL® reagent, and incubated for 5 min at room temperature. Two hundred µl of chloroform was added to the homogenate and the sample was vortexed for 30 sec. Samples were centrifuged at 12,100 *xg* in a Eppendorf centrifuge 5415 C at 4 ^{0}C. Total RNA was precipitated using 500 µl 100% isopropyl alcohol, washed with 500 µl 75% ethanol, and resuspended in 40 µl Diethyl pyrocarbonate (DEPC)-treated water. Total RNA was quantified in a Nanodrop spectrophotometer (NanoDrop Technologies, Rockland, DE).

RT-PCR was performed on total RNA using the Reverse Transcription System Kit (cat # A3500, Promega, Madison, WI). cDNA was synthesized according to the manufacturer's instructions. One mg of total RNA was denaturated at 65 ^{0}C for 15 min and placed on ice for five min. The reverse transcription reaction mixture containing 5 mM $MgCl_2$; 1X Reverse Transcription Buffer (10mM TRIS-HCl pH 9.0 at 25 °C; 50mM KCl; 0.1% Triton®X-100);

1mM each dNTP; 1 u/µl Recombinant RNasin® Ribonuclease Inhibitor 15 u/µg; AMV Reverse Transcriptase; 0.5µg random primers per microgram of RNA was immediately added to the RNA samples and were incubated at room temperature for 10 min followed by incubation at 42 °C for one hour. To inactivate the AMV enzyme, samples were heated at 95 °C for five min and then placed on ice. cDNA was used as a template for the CP primers described above. PCR products were purified with QIAquick® PCR Purification Kit (Qiagen, Germantown, MD) following the manufacturer's protocol. Briefly, five volumes of PB were added to one volume of PCR sample. The mixture was applied to a supplied DNA binding column and centrifuged for 60 sec at 9,300 *xg*, the column was washed with 0.75 ml of PE buffer, and DNA was eluted by applying 40 µl of 65 °C prewarmed water.

Purified sequence fragments were subcloned into the entry vector pENTER-D/TOPO, which carries two recombination sites, *attL1* and *attL2* for the next cloning step (Figure 1). The cloning reaction was carried out according to the manufacturer's instructions. 500 ng of PCR product was mixed with 1 µg of pENTER-D/TOPO vector and 1 µl of salt solution (1.2 M NaCl, 0.06 M $MgCl_2$). The reaction was incubated for 5 min at room temperature, 2 µl of the solution were added into a vial *E. coli* TOP 10 competent cells (Invitrogen, Carlsbad, CA), and incubated on ice for 30 min. The cells were transformed via heat shock for 30 sec at 42 °C and immediately transferred to ice, 250 µl of room temperature S.O.C. medium (2% Tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10mM $MgCl_2$, 10 mM $MgSO_4$, 20mM glucose) were added and the mix was shaker incubated for 1 hour at 37 °C. Transformed bacteria were placed on LB medium containing 50 mg/L kanamycin sulfate and incubated overnight at 37 °C. Putative colonies were individually selected and cultured in 5 ml of LB broth containing the selective antibiotic. For bacteria DNA extraction, E.Z.N.A Plasmid Mini Kit from Omega Bio-Tek, Inc. (Norcross, GA) was used following the manufacturer's instructions. Briefly, the bacterial cells were pelleted by centrifugation and resuspended in 250 µl RNases solution I. Two hundred and fifty µl of the alkaline-SDS lytic solution II was added and the tube was inverted four to six times to form a clear lysate. To neutralize the solution and precipitate proteins and carbohydrates, 250 µl of solution III was added to the lysate. The lysate was then centrifuged at 10,000*xg* and the supernatant was added to the supplied binding column. The column was centrifuged for one minute at maximum speed in a microcentrifuge. The column was then washed with 700 µl of DNA washing buffer-containing ethanol and spun for 60 sec at maximum rpm. The DNA was

eluted from the column with 40 µl of 65 °C prewarmed water followed by 60 sec of centrifugation at maximum rpm. Bacterial DNA PCR and restriction endonuclease digestion were used to confirm the presence of the CP fragments. DNA sequencing was performed by KSU DNA Sequencing and Genotyping Facility to determine the correct orientation of the inserts as well as verifying proper amplification of the sequences derived from the CP of each virus.

To produce the plant expression vector, the CP fragments were independently cloned into pANDA mini vector (Daisuke Miki and Ko Shimamoto, 2003; Figure 1) by means of a homologous recombination reaction LR clonase (Invitrogen, Carlsbad, CA). This reaction between the entry and destination vector allowed the insertion of the identical CP fragments in two regions defined by the recombination sites *attB1* and *attB2*. Consequently the *gus* linker is flanked by the two identical copies of the CP in opposite directions. The transformation procedure was completed using 50 µl of heat shock JM109 competent cells (Promega) transformed with the 25 ng of LR reaction containing the pANDA vectors. The bacteria were incubated on ice for 30 min, heat-shocked at 42 ℃ for 90 sec and placed on ice for 2 min. Five hundred µl of S.O.C. medium was added to the transformed bacteria and incubated for 45 min at 37 ℃ while shaking at 250 rpm. The bacteria were plated in LB medium with 100 mg/L ampicillin and incubated overnight at 37 °C. Colony PCR was performed to confirm the presence of the gene fragment in both recombination sites of pANDA mini and sequencing was required to confirm correct insertion of the gene fragment.

Plant Transformation

Plant material

Immature seed, 10 to 14 days post anthesis of the *Triticum aestivum L*. cultivars Bobwhite and Fielder were harvested from greenhouse-grown plants and surface sterilized with 20% bleach and 0.04% Tween-20. Excised embryos were cultured on callus induction medium CM4 (Table 1) in the dark at room temperature (Figure 2.A). The embryos were oriented on the medium so that the embryo axis was in contact with the medium (Figure 2.B). After one week, organized callus on the scutellum was selected for bombardment. Prior to bombardment the

embryos were air-dried for one hour in laminar flow hood in order to decrease the osmotic pressure of the cells.

Bombardment

Wheat transformation was performed through biolistic particle delivery system as described by Anand et al, (2002). Embryos were co-bombarded with the vector pAHC20 containing the ubiquitin promoter, the RNAi construct containing the target gene for either TriMV or WSMV, and *bar* herbicide resistance, which confers ammonium glufosinate resistance (Christensen and Quail, 1996; Figure 4). Bombardment experiments were performed using helium at 4136.85438 millibars of gas pressure under -1 930.53204 millibars vacuum conditions. Fifty mg of tungsten particles were sterilized, washed, and resuspended in 500µl of sterile water of which 25 µl of resuspended particles were with coated with 5µg of the RNAi plasmid DNA and equal amount of the co-bombardment plasmid. The DNA was precipitated with 25 µl of 2.5 M of $CaCl_2$ and 10 µl of 100 mM spermidine. The emulsion was kept on ice for 4 min and 50 µl of supernatant was removed, 2 µl of the remaining mix was used for each bombardment. Five independent assays using 180 embryos in each experiment was used for each plasmid construct.

Tissue culture

One week after bombardment, the embryos were subjected to a two-week cycle on CM4 containing 5 mg/l of ammonium glufosinate. In the following culture cycle the embryogenic calli were transferred onto a CM4 medium containing 10 mg/l of ammonium glufosinate and incubated for two two-week cycles. Regeneration cycles were carried out in light cycles of 16h day: 8h night at 23 °C. Initially, the cultures were transferred to shoot production medium (MSP) containing 5 mg/l of ammonium glufosinate (Table 1) for two weeks (Figure 3D). The cultures were then transferred to shoot elongation medium (MSE) containing 5mg/l of ammonium glufosinate (Table 1) (Figure 3E). Plantlets showing shoot and roots were transferred into 50 ml tube containing 13 ml of MSE medium (Figure 3F). Three to four weeks later, rooted plants were transplanted into peat pots, placed in an enclosed polystyrene container to maintain high humidity (Figure 3G). Over the course of a week the container's lid was slowly removed to harden plants to ambient humidity levels. Two weeks after transplanting into peat pots the

putative transgenic wheat were transplanted into one-gallon pots, transferred into a growth chamber (16h light 23 °C: 8h dark at 18 °C).

Plant Evaluation

Liberty painting

Putative transgenic plants were tested for herbicide resistance. Liberty (AgroEvo USA, Wilmington, DE), ammonium glufosinate aqueous solution (0.2% v/v), was spread on one third of the leaf area, testing a leaf for each tiller. Resistance was determined according to the necrotic damage seven days after treatment (Figure 5).

PCR gene detection

Transgene PCR detection was made separately for each tiller on the T_0 generation due to the chimeric nature of the recovered plants. Genomic DNA (gDNA) from positive Liberty painting plants was isolated using OMEGA EZNA (Omega Bio-tek, inc. Norcross, GA) plant DNA kit and following the manufacturer's protocol. Ten mg of leaf tissue was collected and ground in a microcentrifuge tube with liquid nitrogen then 600µl of P1 and 4µl RNAse A were added. The samples were incubated at 65^0C for 5 min the 140µl of buffer P2 was added then mixed by vortex and centrifuge at 10,000 x*g* for 10 min, the supernatant was transferred in to a new microcentrifuge tube and mixed with buffer P3 and one volume of absolute ethanol. The mixture was applied into a column and washed with 650 µl of wash buffer. gDNA was eluted with 40 µl of pre-warmed water. For PCR reactions, 200 ng of gDNA was used per reaction.

For the T_2 generation of transgenic material, a high throughput DNA extraction method was used (Allen et al., 2006). Three to six cm^2 of leaf tissue samples were collected and placed in a 96-format microtiter plate (128 x 86 x 42mm). The tissue samples were stored at -80^0C for a short time and then lyophilized for two days. Two steel beads were added to each well and plates were shaken for five min on a Fleming Grey Model C paint shaker (Certified Technology Inc., Niagara Falls, NY). Six hundred µl of lysis buffer (500mM NaCl, 100mM TRIS, 50mM EDTA, 5.0 of sodium bisulfate, 0.1% ascorbic acid, 2% Polyvinylpolypyrillidone) were added to the ground tissue and the plates were vortexed for four min. Plates were incubated for 1 hour at 65^0C and then centrifuged at 3000 for 10 min. DNA was precipitated by centrifugation after the

addition of 50 µl of 10 mM NH_4Ac and 400µl isopropanol to the supernatant. The resulting pellet was washed with 400 µl of 70% ethanol and resuspended in 100 µl TE buffer.

PCR analyses were carried out to identify the presence of *bar* gene from pHC20 as well as the specific RNAi CP transgene. Each PCR reaction contained 10xThermoPol Buffer (20 mM TRIS-HCl 10 mM $(NH_4)_2SO_4$, 10 mM KCl, 2 mM $MgSO_4$; New England Biolabs, Ipswich, MA), 0.2 mM of each (dNTPs), 25 pmol of each primer (Table 2), 1.25 u *Taq* DNA polymerase (New England Biolabs) and 200 ng of template DNA. Amplification conditions were five min at 95^0C; 30 cycles of 30 sec at 95^0C, 40 sec at 60^0C and a final extension cycle of at 72^0C. 15 min.

To determine transgene presence, two separate PCR reaction were carried out, i. to test the antisense arm using the *gus* linker reverse primer and the CP specific reverse primer and ii. to test the sense arm using the *gus*-linker forward primer and the specific CP reverse primer. The amplification conditions used for both reactions were; five min at 95 ^{0}C; 30 cycles of 30 sec at 95 ^{0}C, 30 sec at 58 ^{0}C for 45 sec at 72 ^{0}C and a final cycle at 72 ^{0}C for 10 min. The PCR products were run on 2% agarose gel in a electrophoresis box with 1X TAE buffer (50X 242g TRIS base, 57.1 ml acetic acid, 100ml 0.5 M EDTA pH 8.5), at 100 V for 35 min and stained with 0.033 ng/ml ethidium bromide for 15 min. Gels were visualized with a UV light box and photographed with a digital camera and Kodak 1D image analysis software.

Southern Hybridization

Genomic DNA (gDNA) was extracted from 100 mg of leaf tissue. Tissue was ground with liquid nitrogen, and mixed with 500µl of extraction buffer (1.5 g Glycine NaOH, 50mM NaCl, 10mM EDTA,2% SDS, 2.0 g Sodium lauryl sarcosine) and 500 µl phenol: chloroform: isoamyl alcohol 24:25:1. Samples were incubated 10 min in the vertical shaker and centrifuged at 12,100 x*g* for 10 min. The aqueous phase was transferred into a new microcentrifuge tube and DNA was precipitated with one volume of 100% isopropanol and 1/10 volume of 3M NaOAc, the pellet obtained was washed with 500 ul of 70% ethanol and resuspended in 40µl of 1X TE buffer.

For each sample each, 30 µg of gDNA was digested with 50U of *Kpn*I and the reaction was carried out at 37 ^{0}C for 24 hours. A positive control of 1 µg plasmid DNA was digested with *Kpn*I, the reaction was incubated for 1 hour at 37^{0}C, inactivated at 65^{0}C, and diluted to obtain a final concentration of 5 pg/µl. The samples and positive control were loaded in a 0.8% agarose gel and separated for 24 h at 35 volts. Gels were stained with ethidium bromide and visualized in the UV box to verify proper digestion and equal loading of DNA. The gels were depurinated in two volumes of 0.25N HCl and denatured in 0.4 M NaOH for 20 min, the N^+ membrane (Amershad Biosciences, Piscataway, NJ) was equilibrated in the same solution for 10 min. The DNA in the gel was transferred using semi-dry capillary method. After transfer the membrane was washed in 2xSSC for two min. (Sambrook and Russell, 2001).

Blots were pre-hybridized with 20 ml of hybridization buffer (5x SSC, 0.5% SDS, 20 mM Na_2PO_4) and 500 µl of denatured ssDNA and incubated at 65 ^{0}C over night. Portions of the CP and *bar* gene were used as probes (Figure 4). The radioactive labeling of the segments was made using 50-100 ng probe DNA (boiled for 10 min and set on ice for 10 min), 5X labeling buffer (500 mM TRIS, 35mM MgCl, 5.0mM DTT), 1.5 µl random primers, 5 µl of dNTP-dCTP (3mM each), 1.0 µl Pol2 Klenow fragment and 5.0 µl $dCTP^{32}$. The labeling reaction was carried out overnight at room temperature. The probe was purified using a NICK^{TM} column (GE Healthcare, UK) following manufacturer's instructions. The probe was denatured at 95 ^{0}C for five min and kept on ice for five min before being added into the hybridization tube. The membrane was hybridized at 65 ^{0}C overnight. The membrane was then washed twice with washing buffer 1 (5% SDS 4mM $NaHPO_4$) for 30 min and washing twice with buffer 2 (1% SDS 4mM $NaHPO_4$) for 30 min. The membrane was exposed a phosphor screen for 24 hours and scanned with the Storm ^{TM}840 (Sambrook and Russell, 2001).

T_1 and T_2 Generation Evaluation

Seeds from T_0 positive plants were collected and germinated. DNA was extracted from plants and PCR analysis was used to confirm the presence of the gene of interest. Total RNA was extracted as described above to determine the transgene expression. RT-PCR was performed on

total RNA using the Reverse Transcription System (catalog # A3500) from Promega. cDNA was synthesized according to the manufacturer's instructions as previously described in the cloning process. The cDNA was used as a template for two PCR reactions; the first one to determine the expression of the *gus* linker using *gus* sense and anti sense primers to amplify a fragment of 636 bp in length from the gus linker. The second reaction was made to determine the expression of the CP fragment on WSMV or TriMV RNAi transgenic plants. An additional PCR was performed using primers designed from an endogenous gene, *alpha* tubulin (Li et al. 2005), to verify the amount of the cDNA and discard any eventual DNA contamination. A PCR product of 500 bp as opposed to the expected 400 bp fragment would indicate presence of DNA contamination in the sample.

Bioassays

Bioassays were carried out on T_1 and T_2 generation plants at the three-leaf stage after they were tested for the presence of the transgene and its expression. Mechanical inoculation was done using 1 g of fresh leaf tissue infected with TriMV or WSMV, blended with 20 ml of 0.02M Phosphate buffer (2.74 g KH_2PO_4, 3.48 g $KHPO_4$ in 1L water) and filtrated through cheesecloth to remove plant tissue. The filtrate was added to de-Vilbiss atomizer sprayer along with 0.3 g of 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 controlled-environment chamber (16:8 l:d cycle, 500 μE m^{-2} s^{-1}) at 19 ^{0}C. Leaf samples were taken 14 to 21 days post-inoculation and stored at -80 ^{0}C until used ELISA or RT-PCR.

ELISA T_1 and T_2 generation

Virus presence was determined by indirect enzyme-linked immunosorbent assay (ELISA) according to prescribed protocol (Agdia). Samples from transgenic material, nontransgenic inoculated wheat, and uninfected samples were used in the assays. 100 µl of indirect coating buffer (15 mM Na_2CO_3, 34.8 mM $NaHCO_3$, 3 mM NaN_3) were pipetted into the ELISA plate

wells. Two hundred mg of leaf tissue were ground with 2 ml of indirect extraction buffer (1X PBS, 10g polyvinyl pyrrolidone; Agdia). One hundred µl of the extracts were added into each well and shaken at room temperature on an orbital shaker in a humidity bag for one hour. The liquid was removed from the wells and the plate was washed with 1X PBST buffer (0.13 M NaCl, 1.4 mM KH_2PO_4, 8.1 mM Na_2PO_4, 2.7 mM KCl, Tween 20). The specific rabbit antiserum (provided from KSU Plant Diagnostic lab) for each virus was diluted 1:100 in the extract of healthy wheat ground in indirect extraction buffer as above. Afterwards 100 µl of enzyme substrate (PNP) were dispensed into the wells and shaken in dark for one hour until they were read at 405 nm on a plate reader (Agdia).

siRNA Detection

Small RNA fraction and RNA hybridization

For small RNA detection, total RNA was extracted from 1g of leaf tissue using TRIZOL reagent. Low molecular weight RNA was obtained using DNA/RNA extraction kit (QIAGEN) according to the manufacturer's 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 850 µl of ice-cold isopropanol and centrifugation at 15,000 xg for 30 min at 4 ^{0}C. Low molecular weight RNAs were washed with 75% ethanol, air dried, and resuspended in 50 µl of DEPC water.

Low molecular weight RNA was separated by electrophoresis on 15% polyacrylamide gel (30% acrylamide 5ml, urea 4.2g, 10XTBE 0.5ml, 10% 80µl and TEMED 6 µl). Samples were denatured by the addition of one volume of deionized formamide and heated at 65^{0}C for 5 min then kept on ice for 5 min. Single strand RNA loading dye was added and the mixture was loaded in to the gel. A mix of 2 µl of the specific primers for each viral CP segment was used as a positive control. Gels were run for one hour at 180 volts. An siRNA marker (Biolabs, San Diego, CA) was loaded to indicate sizes of 15, 21, 27 bp. To determine the position of each band the marker was removed before blotting stained in ethidium bromide and measure with a ruler.

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

washed with 2X SSC buffer for 2 min; UV cross-linked and air-dried overnight. For Pre-hybridization and hybridization, 6ml of pre-warmed ULTRhyb-oligo buffer (Ambion, Austin, TX) was used. To prepare the probe, primers were designed from the cloned sequence to amplified segments no bigger that 100bp (Table 2), the PCR products were gel extracted, a mix of the small segments obtained was radioactively labeled (10.0 µl) denatured at 95 min for 10 min and then added into the hybridization tube. Hybridization was carried out at 42 ^{0}C overnight in the rotating hybridization oven. The hybridization buffer was discarded and the membrane was washed with 1X SSC, 0.8% SDS for 15 min at 42 ^{0}C and 1X SSC, 0.8% SDS for 15 at 42^{0}C. The membrane was placed in the cassette and exposed to a phosphor screen for two days and then scanned with the Storm^{TM} 840.

Segregation analyses

PCR transgene detection data of T_1 generation of plants was used for segregation analyses. To estimate the pattern of heritage of each line Chi-square goodness-of-fit tests were performed to establish whether the observed segregation ratios of CP-positive plants to negative plants fit the expected Mendelian 3:1 for integration at a single locus or two loci integration 15:1 (Table 14).

Data Analysis

For data analyses two by two contingency tables (Tables 15-18) were constructed from the data obtained in transgene PCR detection and the ELISA result. Each table was made independently for each construct and each generation of plants.

Chi-square analyses of independence were used to determine the association between the transgene presence and the ELISA result. The Yates continuity correction was applied to these tables.

Posterior analyses were performed on the set of data that showed lack of independence in the analyses for transgene presence. Two by two contingency tables (Tables 5.4- 5.6) were constructed to determine the relationship between the expression of transgene (RT-PCR data) and the presence or absence of virus (ELISA results).

Results

Expression of WSMV CP RNAi vector:

Production of transgenic plants (To)

Approximately 900 wheat callus explants were bombarded in five independent biolistic transformation experiments. One week after bombardment the calli were transferred to selection media (CM4 media containing 5 mg/l glufosinate) for a two-week cycle, and then transferred to CM4 + 10 mg/l glufosinate for another two-week cycle. During this selection process, the calli did not show major morphological changes except an increase on size and, in few cases, roots were produced. The selection agent suppressed growth of the majority of the calli (Figure 2). Under regeneration conditions on MSP media, also containing 10 mg/l glufosinate, the calli became more organized and distinguishable shoot and leaves appeared followed by root growth (Figure 3). However, some in cases, roots appeared before shoots. The regeneration cycles were completed in MSE+5G media where the shoots were elongated and roots developed more vigorously. One hundred and thirty-six plants, regenerated under glufosinate selection were transferred to soil and kept under high humidity conditions, in polystyrene containers. During this adaptation process, 12.5% mortality was observed. Surviving putative transgenic plants were transplanted into one-gallon pots after the wheat plants grew to the three-or four-leaf stage.

Analyses of T_0 plants

Liberty painting helped screen putative transgenic plants and was performed one week after transplanting to the one-gallon pots. Each tiller of all recovered plants was evaluated separately as one leaf per tiller was tested. If the leaf was susceptible, it would become chlorotic and then turn brown in about ten days (Figure 5). Resistant leaves would remain green. Some plants exhibited an intermediate phenotype and were considered positive until molecular analysis could provide a definitive answer. Thirteen liberty-positive plants were obtained for the WSMV CP hairpin construct. Due to the low selection pressure of the glufosinate some calli were chimeric. As a result, some of the regenerated plants contained both herbicide-resistant and susceptible tillers. The herbicide-susceptible tillers were removed. DNA was isolated from putative Liberty-positive plants and used as a template for *bar* gene primers (from pHC20 co-bombardment vector). Eight of the putative liberty-positive plants were confirmed to contain b*ar*

gene (Table 2; Figure 7). In most of cases, both co-bombardment vector and hairpin construct were detected in the same plant sample. Seeds of each tiller from T_0 plants were independently collected.

T_1 Transgenic plants

Initial analyses of T_1 progeny were carried out on five different lines derived from T_0 PCR positive plants: Lines 34, 35, 110 147 and 195. Tillers from each line were independently evaluated (Table 4). PCR analyses, using specific primers for the sense and antisense fragment of the WSMV CP hairpin construct, determined the number of positive plants of each tiller. RNA was extracted from plants containing the transgenic insert and the cDNA was used as a template for two separate PCR reactions to determine mRNA expression of CP and *gus* linker. RT-PCR experiments demonstrated the expression of CP in almost all the samples containing the transgene. On the other hand, relative low mRNA expression of *gus* linker was found only in lines 147 and 195, the other lines did not show any signal of *gus* linker expression. *Alpha* tubulin was used as an internal control to corroborate the quality of the cDNA used for amplification. Another observation was made on the size of the bands obtained in the plants from lines 195 and 110. These bands both sense and antisense, obtained gDNA PCR transgene detection, were smaller that the obtained from the positive control (initial pANDAmini vector containing the fragment of WSMV CP, used for the transformation experiments). Sequencing of these segments indicated a deletion in the sequence of *gus linker*.

Preliminary Bioassay

In initial experiments, plants from four transgenic events containing the hairpin structure (lines 34-E, 110-B, 147, 195A) were subjected to virus inoculation. Twenty-one days after inoculation samples from the youngest leaves were taken for molecular analysis and symptoms were recorded. Typically infected plants showed a discontinuous mosaic pattern parallel to the leaf veins (Figure 6A and B), and plants from line 147 also exhibited a stunted phenotype (Figure 6C). RT-PCR analyses of infected plants confirmed that plants exhibiting symptoms were infected with WSMV. These analyses were made with primers for the cylindrical inclusion (CI) protein of WSMV in order to avoid the amplification of transgene CP mRNA. Phenotypical data indicted the highest level of resistance among the transgenic plants tested were from line 110 tiller B. The other lines tested, such as 34, 35 and 147, did not show resistance in most of plants tested in any of their tillers. These results were confirmed with RT-PCR (Table 5).

A second analysis was carried out in five tillers of lines 462 and one tiller of line 147. Plants were tested for presence of the sense and antisense transgene sequences using PCR analysis (Table 6). Four out of five tillers evaluated in line 462 were confirmed to contain the WSMV construct although deletion of one of two sides of the construct was also observed in few of these plants. PCR of the plants from tiller 147 confirmed that the transgene was intact. mRNA expression analyses (RT-PCR) of CP for line 462 showed no clear expression of CP nor *gus* linker. Analyses of line 147 showed expression consistent expression of *gus* linker but CP mRNA was not detected.

Bioassays of T_1 progeny

T_1 progeny from lines 462 and 147 were challenged with WSMV by mechanical inoculation and to avoid inoculation failures, a second inoculation was performed one week later. Symptoms were recorded and samples for ELISA were taken twenty days after the first inoculation. According to ELISA values and phenotypical data, resistance was presented in two out of five tillers evaluated in line 462, and was presented in the plants containing the transgenic CP hairpin construct. In the plant evaluated for line 147-a1, a consistent resistant response was observed in 8 out of 12 plants, four susceptible plants in this line showed a delay in symptom development and low virus titer in ELISA assays (Table 7). The negative control non-transgenic plants showed 100% virus incidence.

T_2 transgenic plants analyses

For T_2 progeny analyses, ten seeds from each line 462-B2, 462-D3, 462-E2, 110-B4, 110-B8, 147a1-1 and 147a1-4 were germinated and evaluated for transgene presence, mRNA expression and challenge to determine virus resistance (Table 8). PCR analyses of plants from 462-B2, 462-D3 and 462-E2 showed the insertion of both of the sides of the hairpin construct. In the analyses of line 147-1, the transgene was not detected in half of the plants evaluated (5 out of 10). Progeny from line 147-4 either did not contain the transgene or only one side of the hairpin construct was present. All the plants from lines 195-A20, A17 and 110-B8, B4 lost the antisense arm of the construct (Figure 8). An additional PCR was carried out on the gDNA of 110 and 195 lines. Using the sense and anti-sense primers for the amplification of *gus* linker, two bands were found, one corresponding to the size of the control (initial bombardment vector) and another with lower size of approximately 200bp.

RT-PCR analyses to confirm mRNA transgene expression was made on plants containing either the complete or one side of the construct *gus* linker and CP expression were checked separately. In this generation of plants, not one of the lines showed expression of the *gus* linker. CP expression analyses of plants form lines 462 and 147 did not present CP expression (Figure 9). However, lines 110- B8 and 110-B4 consistently expressed CP message in all plants evaluated. In contrast lines plants from lines 195-A20 and 195-A17, did not express the CP in all the cases where the transgene was present.

Bioassay of T_2 progeny

Mechanical inoculation was performed twice on the T_2 progeny of the nine lines evaluated. ELISA results, performed on samples taken 21 days post-inoculation, indicated that lines 462, 147 and 195-A17 presented the highest number of susceptible plants. All the plants in lines 462- D3 showed virus susceptibility, eight out of ten plants from lines 462-E2, 462-B2, and 147-4 presented virus infection, six out of ten plants from lines 147-1 and 195-A17 displayed virus infection. On the other hand, lines 110-B8, 110-B4, and 195-A20 exhibited the highest number of resistant plants. Five out of ten plants of lines 110-B4 and 195-A20 showed no virus infection. No infection was observed in progeny from line 110-B8 (Table 9).

Expression of TriMV CP RNAi vector:

Production of T_0 transgenic plants

A hairpin construct containing a 272 bp TriMV CP segment and vector pHC20 were co-bombarded in five independent biolistic transformation experiments as previously described above with WSMV CP transformation experiments. During the selection process, the only notable change was an increase in callus size. Shoots began to be produced on calli during the light regeneration cycles on MSP media as expected. The shoots elongated on MSE+5G media where the plants completed the regeneration cycles by producing roots. 176 plants were regenerated under glufosinate selection, transferred to soil and kept two weeks under high humidity conditions. During this period 13% mortality was observed.

Analyses of T_0 plants

Liberty screening was performed one week after transplanting. One week after the painting, positive plants were identified by their ability to remain healthy. Leaves of Liberty-

susceptible plants became pale green to necrotic. Twenty-two liberty positive plants were detected. Some of the plants contained both herbicide-resistant and susceptible tillers and the herbicide-susceptible tillers were removed.

Sixteen positive *bar* gene plants were identified and used for PCR analyses of genomic DNA (Table 3). Of these plants, only ten contained the TriMV CP RNAi construct and were confirmed to contain both sides of the hairpin structure (Figure 10). Seeds of T_0 plants were independently collected.

T_1 Transgenic plants

Analyses of T_1 lines were carried out on two lines, 817 and 201. Every tiller from both of these plants were independently evaluated (Table 10). The results revealed just two deletion events of one of the sides of the construct among the plants evaluated for 201 and three cases for line 817. Low expression of the CP gene and no expression of *gus* linker was observed in line 201. Expression analyses of line 817 showed CP expression in almost all the plants containing the transgene. On the contrary, very low or no expression of *gus* linker was observed in these same lines.

Bioassays T_1 progeny

The bioassays were performed on two tillers from line 201 and four tillers from line 817. Plants were inoculated twice, one week apart. Symptoms were scored and samples for ELISA assays were taken 21 dpi. Nine out of 13 plants from line 201-A and seven out of 14 plants from line 201-D contained the transgene and were negative for viral presence. All of the plants from tiller C were positive for viral presence and five out seven plants from line 817 tiller D and E and four out of seven plants of line 817 tiller F contained the transgene were negative for virus presence. Seeds were harvested from each evaluated plant and were pooled for T_2 generation analyses (Table 12).

T_2 transgenic plants

The T_2 transgenic plants evaluated were derived from four T_1 plants showing the transgene insertion and viral resistance. Plants 201-A19, 201-A5, 201-D10 and 187-F4 were chosen for these experiments. Ten seed from each line were germinated and tested for transgene presence, expression and viral resistance (Table 12).

PCR amplification experiments failed to detect sense or antisense fragment, the deletion phenomena were abundant in these lines. For instance, the plants evaluated in line 817-F4, four

out of ten plants had lost the insert, two plants displaying no signal in PCR analyses for half of the construct and four plants holding both sides of the hairpin. This type of transgene deletion was found among all the lines evaluated (Table 12). RT-PCR was performed on these plants to determine transgene expression. Only lines 201-A19 (Figure 11), and 201-D10 showed expression of both TriMV CP and *gus* linker. However, the expression was not consistent in all the plants containing the transgene in these two lines.

T_2 Bioassays

Mechanical inoculation was performed twice on the five lines previously analyzed and at twenty-one days post inoculation, ELISA assays were performed. The results of these assays indicated that the line with the higher number of resistant plants was line 201-A19. Eight out of nine plants evaluated showed negative ELISA results and all the plants in line 817-F4, which showed no disease symptom and were ELISA negative (Table 13). In contrast, plants in line 201-D10 were all susceptible, and line 201-A5 just two out of ten plants showed no viral infection.

Segregation analyses

Chi-square analyses of PCR transgene detection data indicate that lines 34, 110,195 and 462 tiller C fit 3:1, suggesting that the transgene integrations occurred in at single locus, although separate integration also could occurred at closely linked loci. In Lines 147-a1, 462 tiller B and D and 817 have a tendency that indicated 15:1 integration ration, suggesting integration at two independent loci. Even though, the p-values allowed accepting the hypothesis for segregation 3:1 or 15:1 the number of samples evaluated in each case is not large enough to make this test conclusive, it just indicated a tendency among the lines (Table 14).

Data Analyses

Contingency tables were used to determine if there is any relationship between presence of the transgenes and virus resistance. The null hypothesis tested was that the virus infection is independent of transgene presence. The different transgenic lines with the same construct were grouped because i. the number plants per transgenic line are not large enough to perform the statistical method with certain and ii. because the hypothesis is that the presence of the construct regardless of the transgenic line confers resistance to the virus. Chi-square was calculated using Yates-Chi square formula to correct for continuity (Chi-square distribution is continuous) and to prevent over estimation of statistical significance.

For the WSMV CP hairpin T_1 analyses, data obtained for transgene PCR detection and ELISA results from tiller 147-a1 and 462 tillers A, B, C, D, E. were grouped. Two by two contingency table of the WSMV CP RNAi T_1 chi-square analyses indicate association between transgene presence and ELISA result (χ^2 = 7.155612, df =1, P = 0.007473) (Table 15). The chi – Yates continuity correction factor corroborated the lack of independence between these two variables (chi-Yates=5.6954, P= 0.017009).

For T_2 progeny analyses of WSMV CP construct PCR transgene detection and ELISA result data from tillers 462-B2, 462-D3, 462-E2, 110-B4, 110-B8, 195-A17, 195-A20 and 147-a1-4, 147-a1-1 was grouped. Analyses of two by two contingency table of T_2 generation (Table 16) of the same construct, showed lack of independence (χ2 = 6.117515, df =1, P = 0.013385). Chi-Yates continuity correction factor confirm these results (Chi-Yates=0.0288, P= 0.0288).

For TriMV CP RNAi T_1 generation PCR transgene detection and ELISA data from lines 201tillers A and D, and 817tillers C, D and E was grouped. The two by two contingency table (Table 17) of association between transgene presence and ELISA results, Chi-square analyses indicate that the relationship between transgene presence and virus presence was not independent (χ^2 = 4.522506, df =1, P = 0.033452). However, when Chi-Yates continuity correction factor was applied, was not significant (Chi-Yates =3.11308, P= 0.077666) indicating independence between transgene presence and the ELISA results. The same analyses of T_2 RT-PCR and ELISA data from 201 tillers A5, A19, D10 and 862 tiller F4 were combined in the two by two tables (Table 18). Chi-square analyses indicated no association between the transgene presence and ELISAs (χ^2 = 0.080049, df =1, P = 0.777231).

To establish whether transgene expression and virus presence has any relationship were constructed two by two contingency tables from the expression data, obtained from RT-PCR and the ELISA results. These analyses were made on the generations that showed any significance in the two by two contingency tables for association of ELISA and transgene presence, WSMV CP hairpin construct T_1 and T_2 generation and TriMV CP construct T_1 generation

For WSMV CP construct T_1 analyses, RT-PCR and ELISA data from lines 147-a1 and 462 tillers A, B, C, D, E. was grouped (Table 19). Chi-square analyses of two by two contingency tables did not showed association between expression and virus presence(χ^2 = 2.440087, df =1, P = 0.11827). On the contrary the same analyses of T_2 CP construct grouping RT-PCR and ELISA data of plants derived tillers 462-B2, 462-D3, 462-E2, 110-B4, 110-B8,

195-A17, 195-A20 and 147-a1-4, 147-a1-1 (Table 20) showed lack of independent of (χ^2 = 20.79293, df =1, P =5.12E-06). This result was corroborated by Chi-Yates continuity correction factor (Chi-Yates =18.69563, P= 1.53E-05).

For the analyses of TriMV CP T_1 generation construct, the data from lines 201 tillers A and D, and 817 tillers C, D and E was grouped (Table 21). Chi-square analyses of contingency tables did not show association between expression and virus presence (χ^2 = 1.490401, df =1, P = 0.222154).

Other Constructs from WSMV:

Cylindrical inclusion protein

A 296bp fragment from the cylindrical inclusion (CI) WSMV protein was cloned in pANDA mini hairpin vector and co-bombarded with pAHC20. From five independent biolistic experiments, approximately 900 wheat callus explants were bombarded with these two plasmids. One hundred and forty plants were recovered from glufosinate selection on tissue culture processes and enclosed at high humidity conditions. The rate of mortality of plants in the adaptation process was 23.6%. Plants were transplanted into one-gallon pots and liberty-painting screen was performed. Thirteen putative herbicide-resistant plants were identified and were subsequently tested for the presence of *bar* gene and hairpin construct using PCR. Three plants were determined to contain the bar gene, but no plants were positive for either sense or antisense side CI gene of the hairpin construct. The transformation experiments were repeated in three independent bombardments using approximately 540 callus explants. Twenty-nine rooted plants were recovered and transferred into high humidity condition in the growth chamber. During the adaptation process three plants died. The liberty-painting screen resulted in 12 putative herbicide-resistant plants. PCR analyses for transgene detection confirmed two plants containing the *bar* gene. However, consistent with previous experiments, no plants contained the CI RNAi vector. This may indicate an inhibitory effect of the CI RNAi vector expression in wheat growth or development.

Eukaryotic translation initiation factor G

A fragment of 517 bp form the host translation initiation factor G (GenBank Accession #EF190330.1) was cloned in pANDA mini and co-bombarded with pAHC20 in five independent biolistic experiments using approximately 900 wheat callus. Embryogenic calli was transferred

into the selection cycles where tissue increased. However, during the regeneration cycle, tissue culture tissue did show morphological differentiation and in many cases, even when leaves were formed roots were not produced. Seventy-two plant generated under glufosinate, were enclosed at high humidity conditions. The rate of dead plants the adaptation process was 33.3%. Plants were transplanted into one-gallon pots and were screened for Liberty resistance one week later. Seven herbicide-resistant plants were identified and six of these lines were confirmed by PCR to contain the *bar* gene. PCR analyses also confirmed the presence of the hairpin construct in three lines of the *bar*-positive plants. These plants are currently being grown to maturity.

Eukaryotic translation initiation factor F

A fragment of 298 bp from the host eiF(iso)4F (GenBank Accession #WHT l F4F28A) was cloned in pANDA mini and bombarded in five independent biolistic experiments using approximately 900 wheat callus. Embryogenic calli was transferred into the selection and regeneration cycles, where morphological differentiation occurred normally. One hundred and nineteen plant were generated under glufosinate selection, and transferred to high humidity conditions. The rate of dead plants the adaptation process was 20.1%. Plants were liberty-painted these screen established 15 herbicide resistant plants were identified and PCR analyses of these plants determined 7 *bar* gene positive plants and specific primer for the hairpin construct identified three lines. These plants are currently being grown to maturity.

6K2-NaI

A construct containing a segment of 397 bp from WSMV 6K2-NaI protein was designed and transformed into wheat embryos. At this point all the tissue complete the selection cycles. Currently some tissue is still undergoing tissue culture regeneration cycles, a few number of recovered rooted plants have been transferred in to the growth chamber and are being evaluated for herbicide resistance. As of the summer of 2009, two liberty painting resistant plants both containing the hairpin construct have been identified.

Discussion

The results indicated that RNAi targeting either the CP of WSMV or TriMV elicit resistance response in transgenic plants. These results concur with previous studies showing various levels of resistance using the full-length sequence of the WSMV CP (Li et al., 2005) or the viral replicase, NIb (Sivanami et al., 2000) in transgenic wheat. The use of hairpin constructs has been shown to increase the efficiency of posttranscriptional gene silencing (Smith et al., 2000) compared to sense or antisense constructs alone. RNA interference-based gene silencing strategy has been successfully used in wheat functional gene analyses reducing the expression of endogenous genes (Fu et al., 2007).

Plants of WSMV CP hairpin construct T_1 generation used in the preliminary assays contained the CP insert and expressed the transgene in most cases (Table 4). However, after inoculation, only plants from lines 195 and 110 exhibited a significant number of resistant plants (Table 5). Analyses of the second set of T_1 lines determined no expression in most of the plants derived from the different tillers of line 462 (Table 6), few plants in this line were ELISA negative. The resistance response was clearly different between the tillers and present only in some of them. When resistance was found, plants contained the transgene. Nevertheless, transgene presence did not necessarily implicate a resistance response, as was observed in the tiller B of the same line (462) where 100% of the plants containing the transgene showed viral symptoms during their whole life cycle. These results suggest that the lack of resistance in this line was probably due to transgene silencing. On the other hand, all the plants from tiller 147-a1 showed to contain the transgenic insert and expression of *gus* linker but not CP expression in any of the plants evaluated. This line exhibited a delay in symptoms development or no symptoms.

We found clear indication of transgene silencing in tiller B of line 462, which did not show transgene expression or resistance. On the other hand, the lack expression but resistance response was present in some of the plants from tiller D and E in the same line. Assuming that RNAi is eliciting plant viral defense, transgene expression levels could have been strong enough to elicit an effect but too low for our detection methods. Another hypothesis to explain this phenomenon could directly involve RNAi mechanism. The hairpin transgene, which contains

dsRNA is recognized by the dicer like enzyme, inducing the RNAi mechanism (Helliwell & Waterhouse, 2005). Dicer could have processed the mRNA prior to virus inoculation, thus RNA would have become part of the RISC complex and target, preventing the detection of transgene mRNA. Another line that showed evidence of this hypothesis was line 147-1-a where only the expression of *gus* linker was detected, but not the expression of CP. This result could indicate that the transgene mRNA was processed without viral presence, given that the samples used for RNA extraction and posterior cDNA synthesis were taken before virus inoculation. In the case of line 1471-a, the dsRNA formed by the inverted CP fragments was processed. Alternatively, most of plants from lines 34 and 147 that did express CP fragment and sometimes the *gus* linker sequence, but they did not exhibit a resistance phenotype.

A high number of WSMV CP T_2 generation plants lost the resistant phenotype (Table 9); it was the case of almost all the plants derived from line 462. Similarly, plants derived from line 147 lost the expression of *gus* linker and susceptible plants did not show the delay in symptoms development or low virus titer, observed in the susceptible plants of the T_1 generation. These losses suggest transgene silencing could occur during the development of T_2 or late in the T_1 generation and be meiotically transmitted. Previous works in WSMV also generated by the biolistic method, found similar results for resistance in plants expressing the full length of the viral CP. The authors suggest that the number of transgene copies generated for bombardment is the main cause of transgene silencing (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 them (Hammond et al., 2001). However, it was not possible to obtain this information from the southern analyses. The failure of these experiments was probably due to either, the size of the probe used, even though different probes were tested in order to detect the transgene including the CP fragment cloned in the hairpin construct, *bar* gene, and the *gus* plus the CP fragment obtained by PCR, or a problem with the hybridization process including related to buffer. The DNA quality and proper digestion seemed to be correct as was confirmed by spectrophotometer quantification and ethidium bromide staining of the corresponding gel. Additionally radioactive labeled probes showed good activity.

Other phenomenon observed was the partial loss of one arm or the complete construct. This case has been also reported in transgenic lines of wheat, carrying the *bar* gene, which physically lost the transgene in T_3 generation (Srivastava et al., 1996). In *Arabidopsis thaliana,*

the Kanamycin resistant gene was lost (Feldman et al., 1997) and in bean the rep-trap-ren genes from *Bean golden mosaic virus* geminivirus also lost its transgene (Romano et al., 2005). This phenomenon is poorly understood, but has been suggested that it is caused by the genetic instability imposed by stress conditions during tissue culture (Risseeuw et al., 1997). However, this hypothesis could not explain the deletions observed in the T_2 generation (Tables 8, 12). Another explanation for this observation is based on intra-chromosomal recombination that lead to the co-elimination of transgenes; this hypothesis also suggests that 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).

Lower than expected molecular weight bands were observed in PCRs of Lines 110 and 195. Sequencing both arms of these lines established that there was no deletion in the two identical CP fragments of the hairpin construct. The smaller size of PCR products obtained is probably due to a re-arrangement or fragmentation in the sequence of *gus* linker, and it occurred in late T_0 generation or in the development of the seeds for T_1. In theory, this deletion does not affect the formation of the double strand required for the silencing mechanism or if it did both fragment still can elicit the silencing mechanism (Fire et al., 1998). Consistent transgene expression and a resistant phenotype were observed in both T_1 and T_2 generation of lines 110B4 and B8 (Figure 8). This suggests that the observed resistance is a consequence of a homozygosis genotype in T_1 generation parental plants. It also shows a strong resistant phenotype suggesting that the resistance in these lines was stably inherited for the next generation. Various studies have suggested that a transgene dose affect resistance response in plants carry transgene homologous to viral sequences. Homozygous loci are able to confer higher level of virus resistance, and stably transmit the resistance into the next generation (Dinant et al., 1997; Tenant et al., 2001). Importantly, the T_2 generation of these lines exhibited loss of the construct antisense arm indicating that the resistant phenotype observed is not due to the hairpin construct instead, it is the remaining fragment of CP that is eliciting the resistance response, as has been previously reported (Lindbo and Dougherty, 1993). In this case the formation of the double strand that triggers the silencing mechanism is thought to be generated by the virus negative strand or replicative intermediate or by the host RdRp (Beclin et al., 2002). The efficacy of the silencing observed in these lines may depend on the production of the transgene RNA above the threshold level, as has been found in homozygous lines (Dinant et al., 1997; Tenant et al., 2001).

In the T_1 generation lines evaluated for the TriMV construct, 817 and 201 had transgene expression in many of the plants containing the transgene (Table 10). However, several plants did not exhibit a resistant phenotype. In the following generation of plants, many deletion events were observed (Table 12). This indicates that the deletion phenomenon was not caused by the sequence itself. It might be related instead to the nature of the hairpin construct and its stability in wheat plants. Additionally, some of the plants derived from resistant plants, such as line 201A lost the expression and the resistance phenotype (Table 13). These losses are likely caused by transgene silencing. The only line that kept high level of resistance was 201-A19, in this case resistance is correlated with the hairpin construct presence. Line 817 showed also a resistant phenotype, but the fact that many of them did not contain the construct denotes a failure in the inoculation.

No correlation was found between transgene expression data obtained by RT-PCR, and resistance response. Plants presenting no detectable transgene mRNA but resistance response, suggest that the RNAi mechanism is constitutively activated as previously reported (Hyli et al., 2005). In this case, siRNA induced by the hairpin construct targets the corresponding mRNA for degradation and that is the reason why it is not detected by RT-PCR. On the other hand, some plants presented transgene expression but not detectable resistance. There are two possible explanations for this outcome. First, the mRNA is not forming the hairpin (dsRNA) required to activate the system. Second, the hairpin is produced and processed, but the triggered RNA response is not enough to render complete resistance (ELISA negative). It has been found that RNAi in transgenic plants can produce different levels of suppression of gene expression (Fu et al., 2007). It is possible that some level of RNAi activity was present in these plants but it was not enough to cause a distinguishable phenotype from the susceptible plants. Moreover, different levels of susceptibility were not screened.

The RT-PCR analyses allows for the detection of the steady-state mRNA at a specific time point of the transcript life. The amount of mRNA of a transcript at each moment is the result of the balance between the rate of synthesis and rate of degradation (Lindbo et al., 1993). Therefore, if the hairpin mRNA is not detected, the hairpin was not produced or it was produced and later degraded by the RNAi mechanism. Conversely, the detection of transgene mRNA means that there is no degradation, or there is degradation but still unprocessed mRNA is

present. These ideas would explain the contradictory results in the correlation between transgene expression (RT-PCR) and virus resistance (ELISA).

Plants recovered from the bombardment experiments and were PCR positive for the presence of the hairpin construct targeting the eukaryotic translation initiation factors G and F (EiF4F and EiF4G) did not show any apparent morphological abnormality, regardless of their role in protein synthesis (Thivierge et al., 2005). A posterior work to determine the functional roles of EiF4E and EiF(iso)4E in the tobacco plants growth, using antisense down regulation, normal vegetative development of plants showing depletion of either EiF4F or EiF(iso)4E was found. However, when the level of both factors was depleted plants displayed a semi-dwarf phenotype. The explanation to the differential phenotype is the compensatory increase in the of the corresponding factor iso-form plants (Thivierge et al., 2005). Similar result were observed Arabidopsis containing a genetic mutation in the EiF4(iso)E4 that completely inhibited mRNA and protein, these mutants presented no morphological changes or developmental modifications. The absence of differential phenotype in our experiment can be explained by the functional redundancy in this family of proteins (Duprat et al., 2002). Additionally another aspect to take into consideration, the variable quantitative response generated by RNAi (Fu et al., 2007), suggesting that the phenotypes observed in the recovered transgenic plants did not necessarily contain strong reduction of the corresponding elongation factor mRNA. However, T1 and T2 plants have been studied to establish transgene in resistance and any possible effects in growth.

Transformation experiments with the cylindrical inclusion protein did not generate any transformants containing the hairpin construct, even though the number of bombardment for this protein was increased. It suggested that the CI contain any lethal property that resulted in death of the tissue containing the transgene. The CI belong the ‘super family 2’ of RNA helicases (Ref), a large protein family with members from all kingdoms and play important roles in all types of processes in RNA metabolism RNA helicases have been implicated in every step of RNA metabolism, including nuclear transcription, pre-mRNA splicing, ribosome biogenesis, nucleocytoplasmic transport, translation, RNA decay, and organellar gene expression (De la Cruz et al., 1999). Sequence and structural analyses revealed homology among this proteins among the V domian of this protein in related and distant members (Fernandez et al., 1995). An hypothesis that can explain the lack of transformants is based in the RNAi mechanism itself, if the CI helicase contain a sequence homology with one present in wheat, that happened to have

an essential function in plant growth, development or any physiological function, the silencing elicited by the construct would interfere with the endogenous protein. Other explanation for the experiments results could be related the CI sequence used in the experiment that contains some characteristics that inhibits the insertion or alerts the plant repairing mechanism, which would remove the sequence.

Conclusion and future work

The experiments generated different levels of WSMV and TriMV resistance. This can be attributed to either the structure of the hairpin transgene (Smith et al., 2000) or the delivery system. For instance, delivery systems based on bombardment introduce several transgene copies at random integration places. This may result in differential transcriptional activity, which in turn could lead to transgene silencing and expression variability. The use of *Agrobacterium*-mediated transformation to introduce the transgene might enhance expression and transgene stability. Additionally, the transgene stability can be affected by chromosomal process, as well as repair or defense process that end physical transgene deletion. An ideal transgene integration scenario requires two main conditions. First, a few copies of the transgene and second the progeny homozygosis. Despite, hairpin constructs have been shown high gene silencing efficiency, we found that expression of only the sense CP fragment could also achieve resistance.

Next experiments have to be done with the T_3 generation of plants lines 110B and 195A for WSMV CP RNAi construct and 201D19 corresponding to the TriMV construct to determine if the expression and resistance are still stable in this generation or analyze the cause of the loss. 6K2NIa, and the elongation factors transgenic T_1 generation plants need to be analyzed for transgene presence expression and resistance. Future experiments can evaluate the effect of the transgenic plants in the virus vector transmission. Additionally the method of siRNA detection has to be improved, we suggest that the failure of the blotting is cause by type of probe used, we tested series of oligonucleotides spanning the CP fragment, but still an RNA probe be synthesized by *in vitro* transcription of this segments.

Additionally the use of constructs containing multiple virus sequence has been shown have high resistance efficiency (Bucher et al., 2006). The co-infection of WSMV and TriMV is

known to have a worse effect on wheat (Kansas State University research and extension, www.oznet.ksu.edu/library/plant2/ep145.pdf), a construct containing sequence from the CP or another protein can be evaluated for resistance.

Table 1. Wheat tissue culture media used in these studies.

Medium	Application	Composition
CM4	Initiation	MS[1], B5 vitamins[2], 40g/l maltose, 2.2 mg/l picloram, 0.5 mg/l 2,4-D 2 g/l gelrite
CM4 + 5G or 10G	Proliferation and selection	MS, B5 vitamins, 40g/l maltose, 2.2 mg/l picloram, 0.5 mg/l 2,4-D 2 g/l gelrite, 5 or 10 mg/l glufosinate[3]
MSP	Shoot production	MS, B5 vitamins, 40g/l maltose, 0.2 mg/l 2,4-D, 1.90 MES, 100 mg/l ascorbic acid, 2 g/l gelrite, 5 mg/l glufosinate
MSE	Shoot elongation	MS, B5 vitamins, 40g/l maltose, , 1.90 MES, 100 mg/l ascorbic acid, 2 g/l gelrite 5 mg/l glufosinate

All tissue culture media were pH adjusted to 5.7 and autoclaved for 20 min at 120 °C

[1] Murashige and Skoog basal media with macro and micro nutrients (Murashige and Skoog, 1962)

[2] B5 vitamins according to Gamborg et al. (1968)

[3]Ammonium glufosinate was filter sterilized and added after autoclaving

Table 2. Primer sequence and descriptions of sequences used in these studies.

Primer name	Sequence (5'-3')	Annealing Temperature °C	Product size	Description
WSMV- F	CACCAATGCAGGCAAGGACAATGAGCAG	63.7	386 bp	amplification of WSMV CP fragment
WSMV-R	TGCGTGTTCTCCCTCACATCATCT	60.1		cloned in RNAi vector
TriMV-F	CACCGATAGACGATGCGACTGGGCAAAT	63.9	272 bp	Amplify of TriMV CP fragment in
TriMV-R	TCTGTTCCTGTGGTGAAAGCTGGT	60.5		RNAi vector
gus F1	CACGTAAGTCCGCATCT TCA	54.9	216 bp +GOI	Used with the CP specific primers to
gus R1	ATCTCTTTGATGTGCTGTGCC	55.6	353 bp + GOI	determine presence of GOI
gus Sense	CATGAAGATGCGGACTTCCG	50	636 bp	RT-PCR primers to establish *gus* linker
gus Antisense	ATCCACGCCGTATTCGG	52		expression
Ci- F	TCCAGGAATGGGCGTGTGATGATA	63.4	256 bp	Used in RT PCR to determine virus
Ci- R	ACACTAGCATCTCTGCCGAGGTTT	60.1		presence in WSMV transgenic material
$TriMV_2$-R	TCTGTTCCTGTGGTGAAAGCTGGT	60.5	408 bp	Used in RT PCR to determine virus
$TriMV_2$-F	CGGCAGCAAATGGACTTGGATTGA	64.7		presence in TriMV transgenic material
BarABR	CCT GCC TTCATA CGC TATTTATTTGC	58.3	500 bp	Amplification of Bar Gen (Co-
UbiABF	CTT CAG CAG GTG GGT GTA GAG CGT G	64.7		bombardment)
Tub-F	ATCTGTGCCTTGACCGTATCAGG	60	409 cDNA	RT- PCR primers internal control used
Tub-R	GACATCAACATTCAGAGCACCATC	60	500bp gDNA	to determine cDNA contamination

Primer name	Sequence (5'-3')	Annealing Temperature °C	Product size	Description
P1-R	CAAACATTGGAGACA	42.1	77 bp	Amplification of small TriMV CP
P2-F	ATGTTTGACGAAGCG	42.5	68 bp	fragments, use as probe in siRNA
P2-R	CATCCGGTAGGCTAAA	42.9		hybridization.
P3-F	GCCTACCGATGGGTT	51.1	89 bp	P1- R used with TriMV-F and
P3-R	TAAGTGACATCCACT	40.3		P4 used with TriMV-R to amplify the
P4-F	TGGATCTCAATTATT	40	56 bp	segments listed in the previous column.
6K2NaI-F	CACCACTACGATGCTGGTGACACCTTGT	59	384 bp	6K2NaI from WSMV
6K2NaI-R	GTGCCAATGCAACTTCACGAACCT	53		
EiF4F-F	CACCCGCAAATGGAGGCAAATGGACTGT	64.8	298 bp	Wheat eukaryotic initiation elongation
EiF4F-R	TCCACCTCTGCTTGGTTTCTGACT	60.1		factor F
EiF4G-F	CACCTCAGCAGCACCATTGGTATCTCCA	60.3	517 bp	Wheat eukaryotic initiation elongation
EiF4G-R	GCTCGGAGCATTCAACCTCCTCAA	63.6		factor G

Table 3. Summary of T_0 transgenic plants analyses.

Gene Target RNAi hairpin construct	Number of Liberty Painting positive plants	Number of *bar* PCR positive plants	Number of Hairpin construct positive plants
TriMV CP	22	16	8
WSMV CP	13	8	10
WSMV CI	25	5	0
WSMV 6k2-NaI	2	2	2
EiF4F	15	7	3
EiF4G	7	6	3

Table 4. PCR and RT-PCR analyses T_1 CP RNAi WSMV plants.

Tiller	Total plants/tiller	Transgene presence			No. transgenic plants	Transgene expression	
		Hairpin	Half side	Negative		CP	*gus* linker
Line 34							
C	8	0	0	8	0	No	No
D	6	0	0	6	0	No	No
E	10	6	0	4	6	6	6
F	5	2	2	1	4	4	4
Line 35							
F	7	3	2	2	5	5	No
G	5	4	1	0	5	3	No
Line 110							
A	4	4	0	0	4	4	No
B	9	5	0	4	5	5	No
Line 147							
A	9	8	1	0	9	9	7
D	6	2	2	2	4	3	No
195A							
A	**22**	17	**0**	5	**17**	**12**	**6**

Table 5. WSMV detection by RT-PCR of T_1 progeny used in preliminary bioassay**.**

Line- tiller	N° of plant evaluated	RT- PCR virus Presence	
		Positive	Negative
34-E	6	4	2
110 -B	5	1	4
147-A	7	7	0
195-A	7	0	7

Table 6. PCR and RT-PCR analyses of T_1 generation WSMV CP RNAi plants.

Line 462							
Tiller	**Total plants/tiller**	**Transgene presence**			**Number of transgenic plants**	**Transgene expression**	
		Hairpin	**Half side**	**Negative**		***gus* linker**	**CP**
A	10	0	0	10	0	0	0
B	9	8	0	1	8	0	No
C	10	0	2	8	2	0	0
D	10	4	5	1	9	0	3
E	4	1	3	0	4	0	0
Line 147							
Tiller	**Total plants/tiller**	**Transgene presence**			**Number of transgenic plants**	**Transgene expression**	
		Hairpin	**Half side**	**Negative**		***gus* linker**	**CP**
1-a	12	12	0	0	12	12	0

Table 7. Correlations of ELISA results and molecular analyses of T_1 WSMV CP RNAi plants.

Line 462	ELISA Negative plants				ELISA Positive plants			
	Transgene Positive		Transgene negative	Total	Transgene Positive		Transgene negative	Total
Tiller	Express+	Express-			Express+	Express-		
A	0	0	2	2	0	0	8	8
B	0	0	0	0	0	8	1	9
C	0	1	0	1	0	1	8	9
D	2	4	1	7	1	2	0	3
E	0	3	0	3	0	1	0	1
Line 147								
1-a	8	0	0	8	4	0	0	4

Table 8. PCR and RT-PCR of T_2WSMV CP RNAi progeny.

Line	Total plants	Transgene presence			No. transgenic plants	Transgene expression	
		Hairpin	Half side	Negative		*gus* linker	CP
462 B2	10	7	3	0	10	0	0
462 D3	10	7	3	0	10	0	0
462 E2	10	6	1	3	7	0	0
147 -1	10	5	3	2	8	0	0
147-4	10	0	0	10	0	0	0
110 B4	10	0	8	2	8	0	8
110 B8	9	0	9	0	9	0	9
195 A17	10	0	7	3	7	0	5
195 A20	10	0	10	0	10	0	6

Table 9. Correlations of ELISA results and molecular analyses of T_2 WSMV CP RNAi progeny.

Plant	No. of ELISA negative plants				No. of ELISA positive plants			
	Transgene Positive		Transgene Negative	Total	Transgene Positive		Transgene negative	Total
	Express+	Express-			Express+	Express-		
462-B2	0	2	0	2	0	7	0	7
462-D3	0	0	0	0	0	10	0	10
462 -2	0	2	0	2	0	7	0	7
147a1-1	0	4	0	4	0	4	2	6
147a1-4	0	0	2	2	0	0	8	8
110-B4	8	0	0	8	2	0	0	2
110-B8	10	0	0	10	0	0	0	0
195 -17	4	0	0	4	3	0	3	6
195-A20	3	2	0	5	2	3	0	5

Table 10. Molecular analyses of T_1 TriMV CP RNAi plants.

Line 201							
Tiller	**Total plants/tiller**	**Transgene presence**			**No. transgenic plants**	**Transgene expression**	
		Hairpin	**Half side**	**Negative**		***gus* linker**	**CP**
A	16	12	1	3	13	0	13
D	17	2	10	5	12	0	12
Line 817							
Tiller	**Total plants/tiller**	**Transgene presence**			**No. transgenic plants**	**Transgene expression**	
		Hairpin	**Half side**	**Negative**		***gus* linker**	**CP**
C	6	6	0	0	6	1	4
D	7	6	0	1	7	0	6
E	9	7	1	1	8	0	8
F	7	2	5	0	7	0	2

Table 11. Correlations of ELISA results and molecular analyses of T_1 TriMV RNAi plants.

Line 201 Tiller	No. Negative plants				No. Positive			
	Transgene Positive		Transgene Negative	Total	Transgene Positive		Transgene Negative	Total
	Express+	Express -			Express+	Express-		
A	10	0	1	**11**	3	0	2	**5**
D	7	0	1	**8**	6	0	3	**9**
Line 817								
C	0	1	0	**1**	5	0	0	**5**
D	5	0	0	**5**	1	0	1	**2**
E	5	0	0	**5**	3	0	1	**4**
F	0	4	0	**4**	2	1	0	**3**

Table 12. Molecular analysis of T_2 TriMV RNAi CP progeny.

Line	Total plants	Transgene presence			No. transgenic plants	Transgene expression	
		Hairpin	Half	Negative		*gus linker*	CP
201-A5	10	4	2	4	**6**	No	No
201-A19	9	6	3	0	**9**	4	5
201-D10	10	8	0	2	**8**	8	8
817-F4	10	4	2	4	**6**	No	No

Table 13. Correlations of ELISA results and molecular analyses of T_2 TriMV CP RNAi plants.

Plant	No. of negative plants				No. of positive plants			
	Transgene Positive		Transgene Negative	Total	Transgene Positive		Transgene Negative	Total
	Express+	Express-			Express+	Express-		
201-A5	0	2	1	3	0	4	3	7
201-A19	4	4	0	8	1	0	0	1
201 D10	0	0	0	1	8	0	2	0
817- F4	0	6	4	10	0	0	0	0

Table 14. Segregation analyses for T1 generation WSMV and TriMV hairpin construct.

Line/ tiller	Total	Ratio	Observed		Expected		Chi-square		Sum	p-value
			+	-	+	-	+	-		
34- E,F	15	3:1	10	5	11.25	3.75	0.14	0.42	0.56	0.4561
110- A,B	13	3:1	9	4	9.75	3.25	0.06	0.17	0.23	0.6310
147-A,D	15	3:1	13	2	11.25	3.75	0.27	0.82	1.09	0.2967
195-A	22	3:1	17	5	16.5	5.5	0.02	0.05	0.06	0.8055
147-1a	12	3:1	12	0	9	3	1.00	3.00	4.00	0.0455
462- B,D,E	23	3:1	21	2	17.25	5.75	0.82	2.45	3.26	0.0710
462-C	10	3:1	8	2	7.5	2.5	0.03	0.10	0.13	0.7150
201-A	16	3:1	13	3	12	4	0.08	0.25	0.33	0.5637
201-D	17	3:1	12	5	12.75	4.25	0.04	0.13	0.18	0.6744
817-D,E.F	29	3:1	27	2	21.75	7.25	1.27	3.80	5.07	0.0244
147-1a	12	15:1	12	0	11.25	0.75	0.05	0.75	0.80	0.3711
462 B,D	23	15:1	21	2	21.563	1.4375	0.01	0.22	0.23	0.6280
817-D,E,F	29	15:1	27	2	27.188	1.8125	0.00	0.02	0.02	0.8856

Table 15. Contingency table for the relationship between transgene presence and virus presence of WSMV CP RNAi construct T_1 generation lines 147-a1 and 462 tillers A, B, C, D, E. Values represent the combine number of individual plants in the analysis.

		Transgene Presence		
		+	-	Total
ELISA	+	17	17	**34**
	-	18	3	**21**
	Total	**35**	**20**	**55**

Table 16. Contingency table for the relationship between transgene presence and virus presence for WSMV CP RNAi construct T_2 generation lines 462-B2, 462-D3, 462-E2, 110-B4, 110-B8, 195-A17, 195-A20 and 147-a1-4, 147-a1-1.

		Transgene Presence		
		+	-	Total
ELISA	+	38	13	**51**
	-	35	2	**37**
	Total	**73**	**15**	**88**

Table 17. Contingency table for the relationship between transgene presence and virus presence, TriMV CP RNAi construct T_1 generation lines 201 tillers A and D, and 817 tillers C, D and E.

		Transgene Presence		
		+	-	Total
ELISA	+	21	7	21
	-	32	2	32
	Total	**53**	**9**	**53**

Table 18. Contingency table for the relationship between transgene presence and virus presence, TriMV CP RNAi construct T_2 generation lines 201 tillers A5, A19, D10 and 862 tiller F4.

		Transgene Presence		
		+	-	Total
ELISA	+	13	5	**18**
	-	16	5	**21**
	Total	**29**	**10**	**39**

Table 19. Contingency table for the relationship between transgene expression and virus presence, WSMV CP RNAi construct T_1 generation lines 147 tiller a1 and 462 tillers A, B, C, D and E.

		Transgene Expression		
		+	-	Total
ELISA	+	5	12	**17**
	-	10	8	**18**
	Total	**15**	**20**	**35**

Table 20. Contingency table for the relationship between transgene expression and virus presence WSMV CP RNAi construct T_2 generation lines 462B2, 462D3, 462E2, 110B4, 110B8, 195A17, 195A20 and 147a1-4, 147a1-1.

		Transgene Expression		
		+	-	Total
ELISA	+	7	31	**38**
	-	25	10	**35**
	Total	**32**	**41**	**73**

Table 21. Contingency table for the relationship between transgene expression and virus presence, TriMV CP RNAi construct T1 generation lines 201tillers A and D, and 817 tillers C, D, and E.

		Transgene Expression		
		+	-	Total
ELISA	+	20	1	**21**
	-	27	5	**32**
	Total	**47**	**6**	**53**

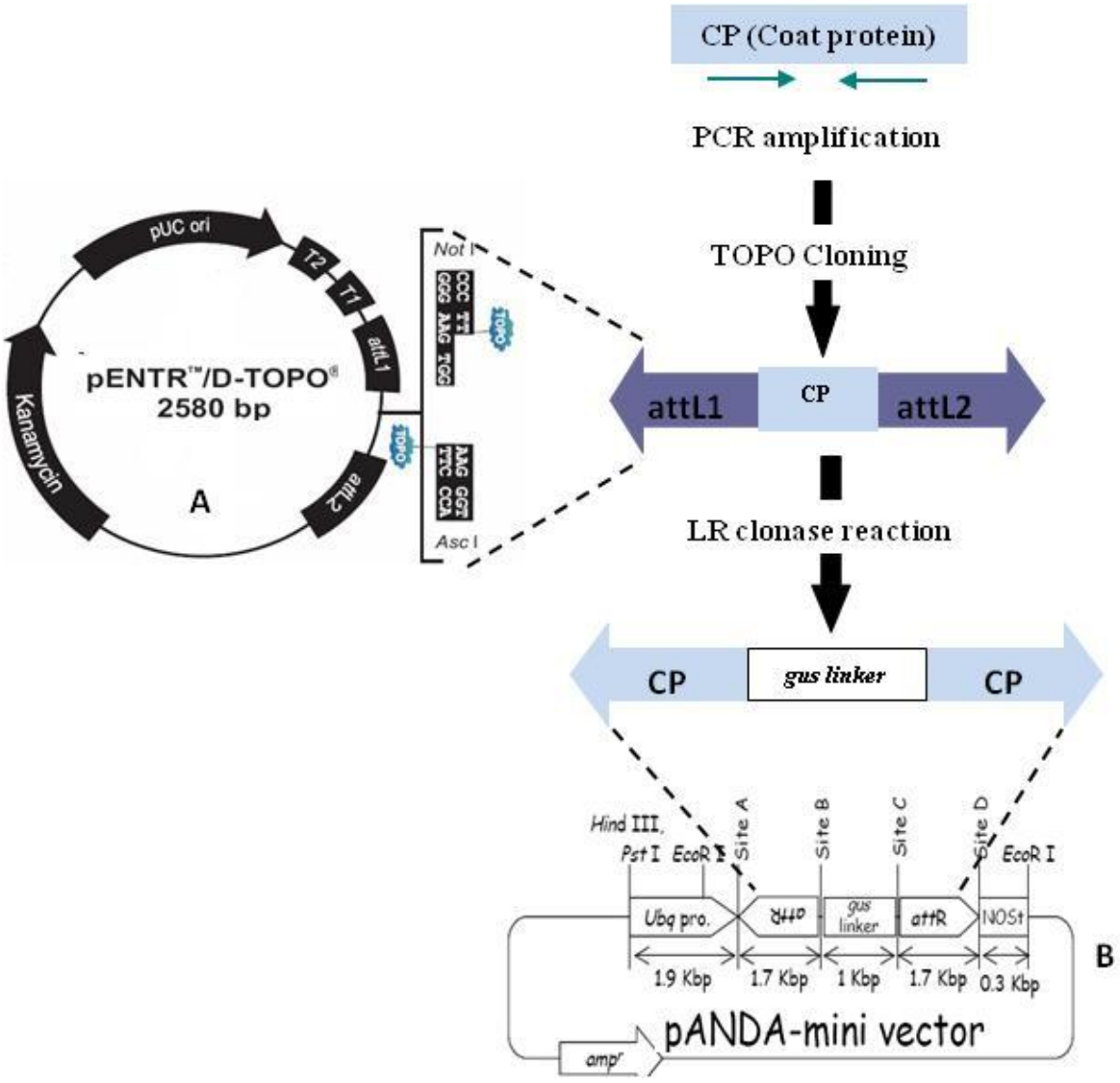

Figure 1.Schematic representation RNAi vector construction. (A) p ENTER/ D-TOPO (Invitrogen, Carlsbad, CA), containing the attL1 and attL2 sites for site-specific recombination of the CP into the expression vector. (B) pANDA-mini (Miki & Shimamoto, 2004) vector with attR sites that allow the insertion of inverted CP sequences flanking the *gus* linker, after homologous recombination. This construction will form a hairpin RNA product when expressed in wheat.

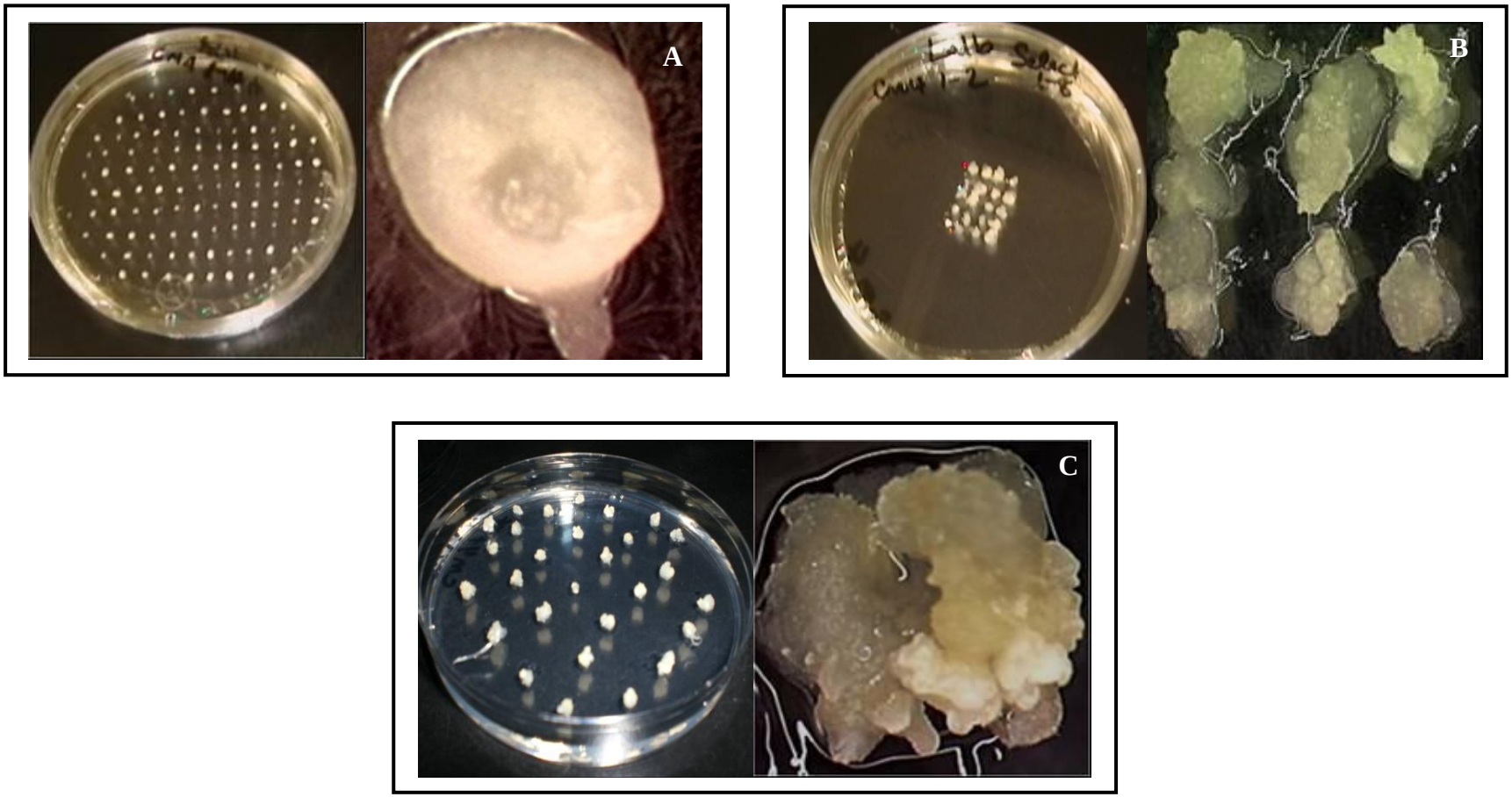

Figure 2. Tissue culture selection cycles in dark. (A) Wheat embryos excised and plated on CM4. (B) Six day-old embryos after bombardment. (C) Embryogenic calli on CM4 medium under glufosinate selection.

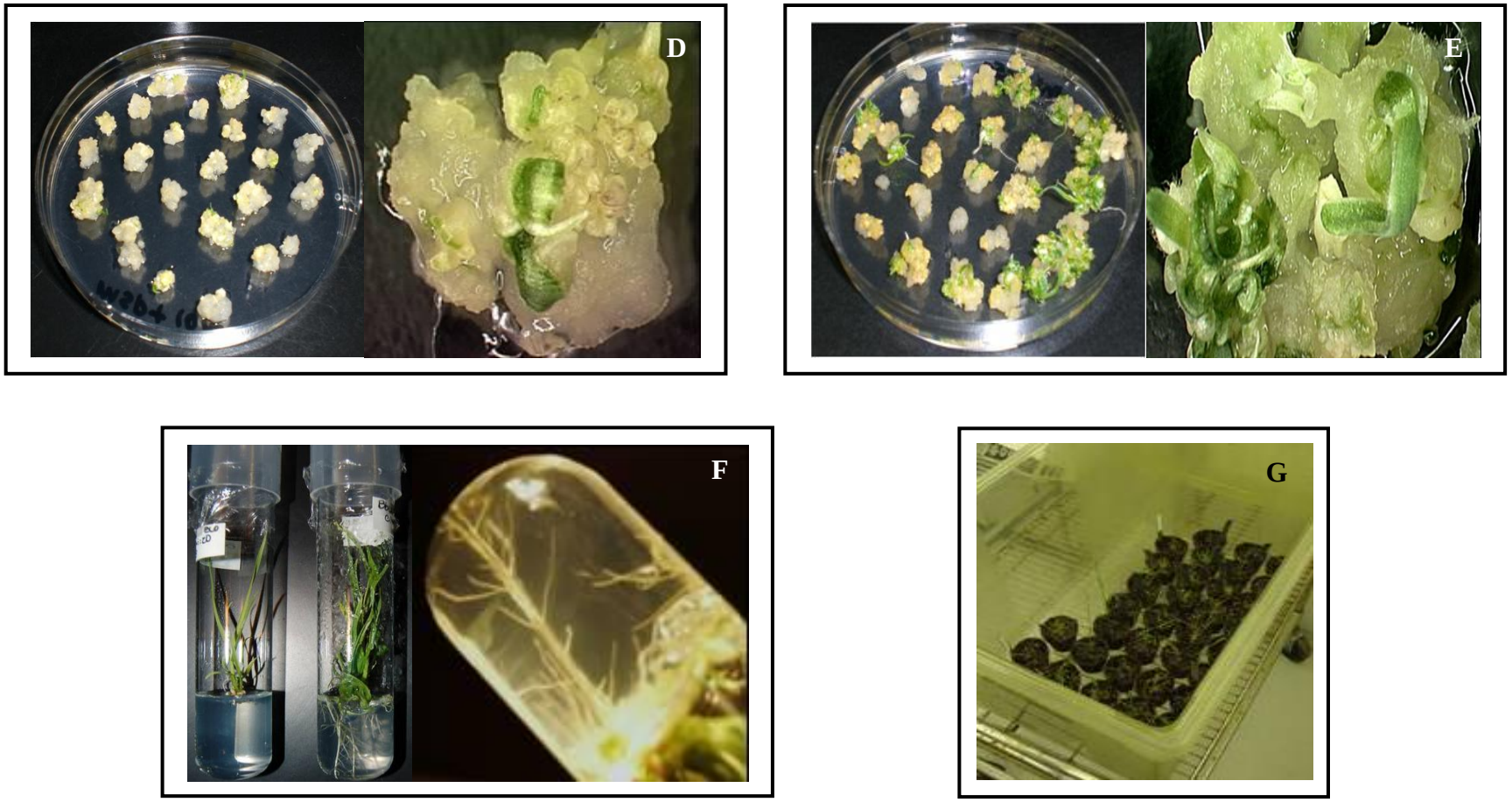

Figure 3. Tissue culture regeneration cycles in light (D) Embryogenic calli on shoot production medium (MSP). (E) Embryogenic calli on shoot elongation medium (MSE). (F) Putative transgenic plants on MSE. (G) Recovered rooted plants in polystyrene container.

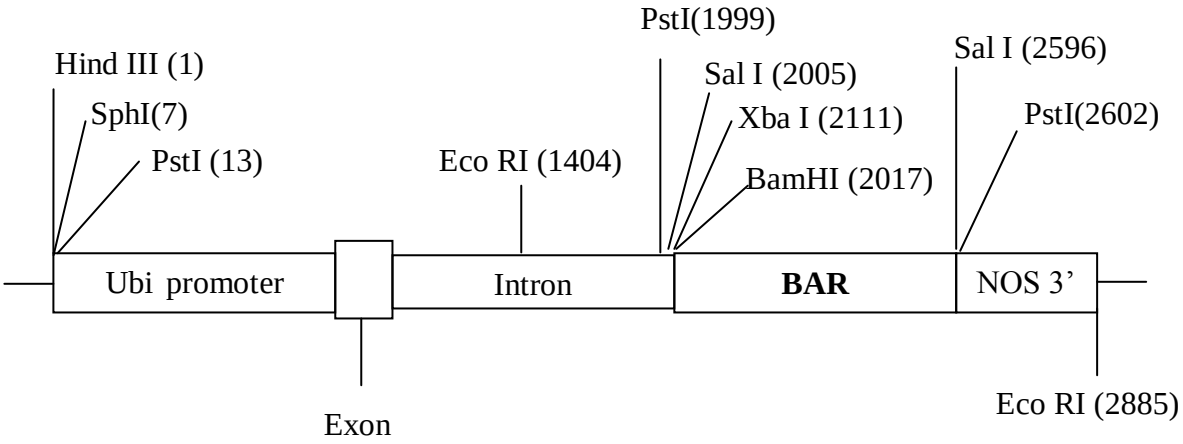

Figure 4. Schematic diagram of pAHC20 containing the *bar* gene for ammonium glufosinate resistance, used in the co-bombardment experiments (adapted from Christensen and Quail, 1996).

Figure 5. Wheat leaves painted with 0.2% Lyberty herbicide. (A) Non-transgenic wheat leaf exhibiting a susceptible phenotype. (C) A leaf from a transgenic wheat plant exhibiting a resistant phenotype to the herbicide.

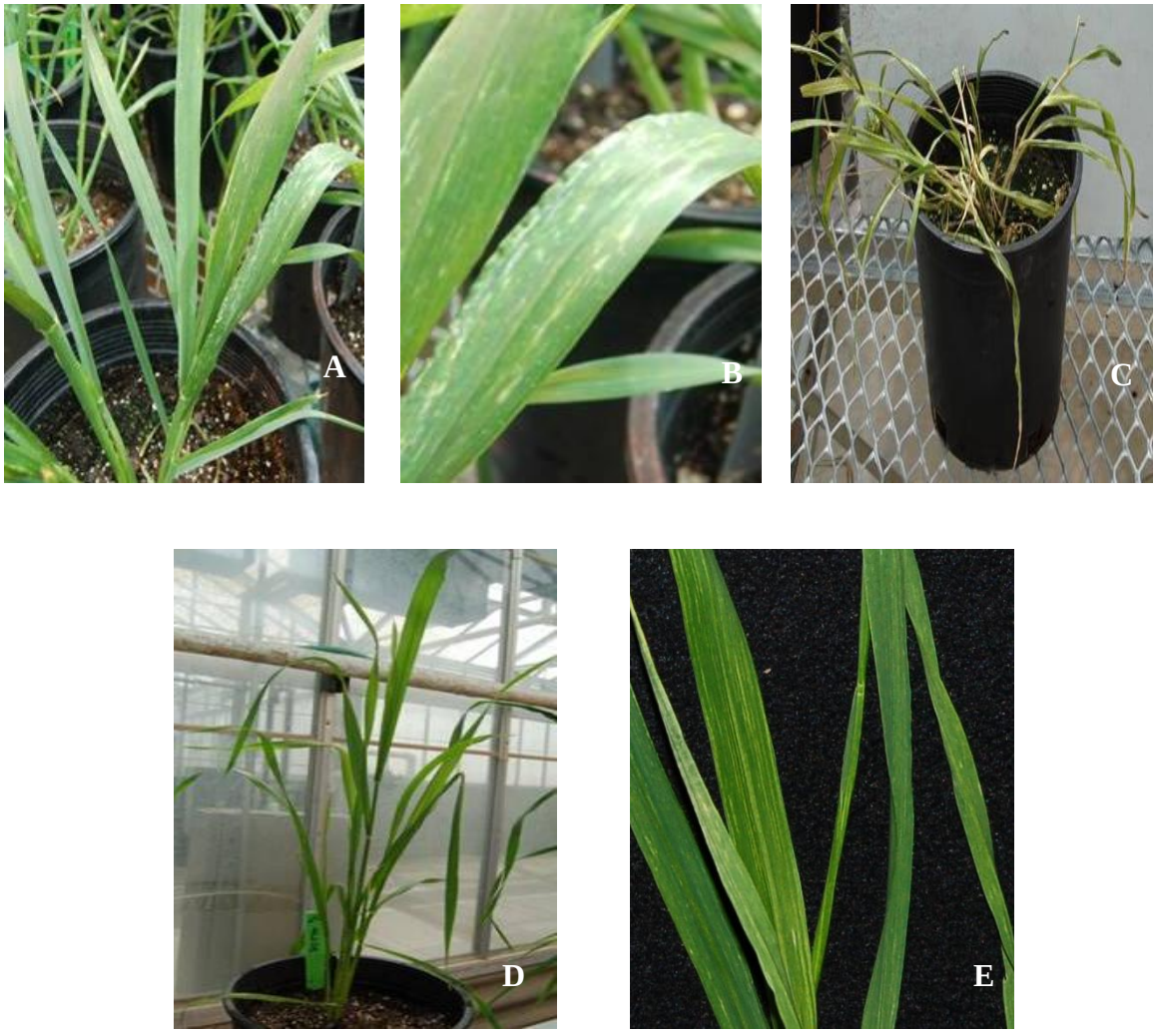

Figure 6. Phenotypes observed in transgenic and non-transgenic. (A-C) Severe infection symptoms, mosaic discontinuous parallel to the leave vein stunt and rosette formation. (C) Final stage of plants with severe infection. (D-E) Symptoms of no severe infection, mosaic presence, but normal plant size and architecture.

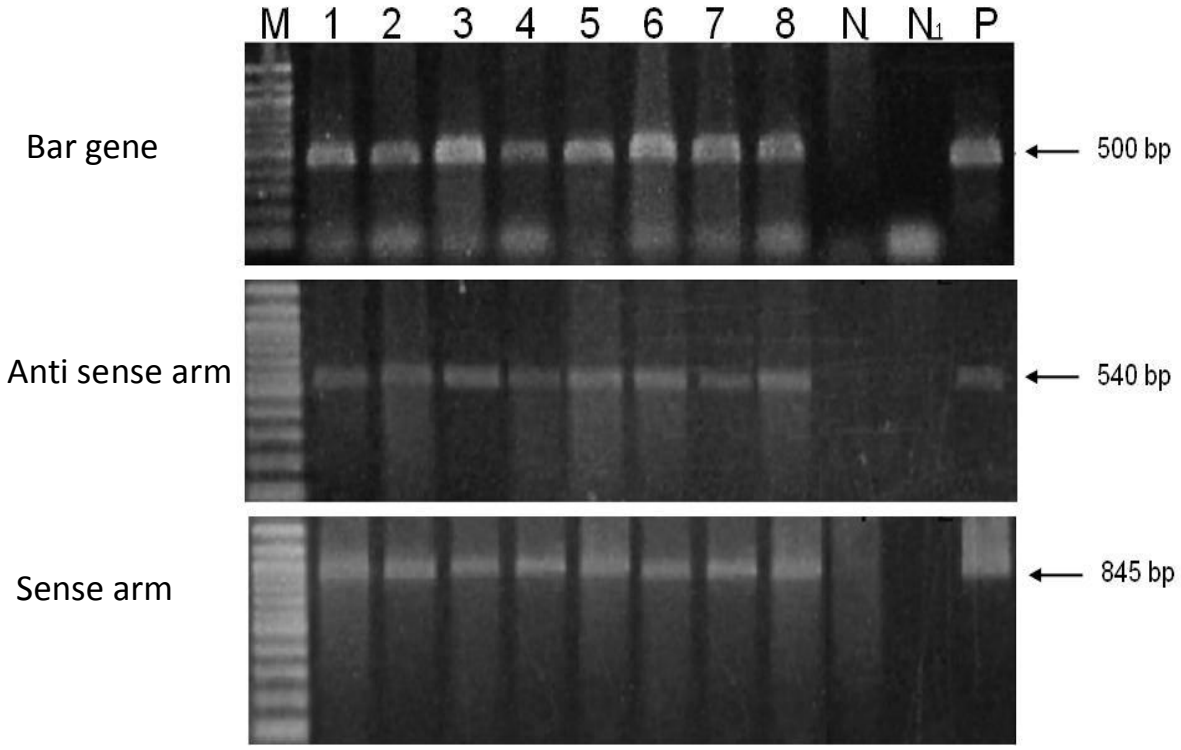

Figure 7. PCR analyses on gDNA of T_0 WSMV CP RNAi transgenic wheat. M, DNA marker; 1-7, seven independent transgenic wheat plants; N_1, non-transgenic wheat plant; N_2, H_2O; P,WSMV CP-pANDA mini (5 pM).

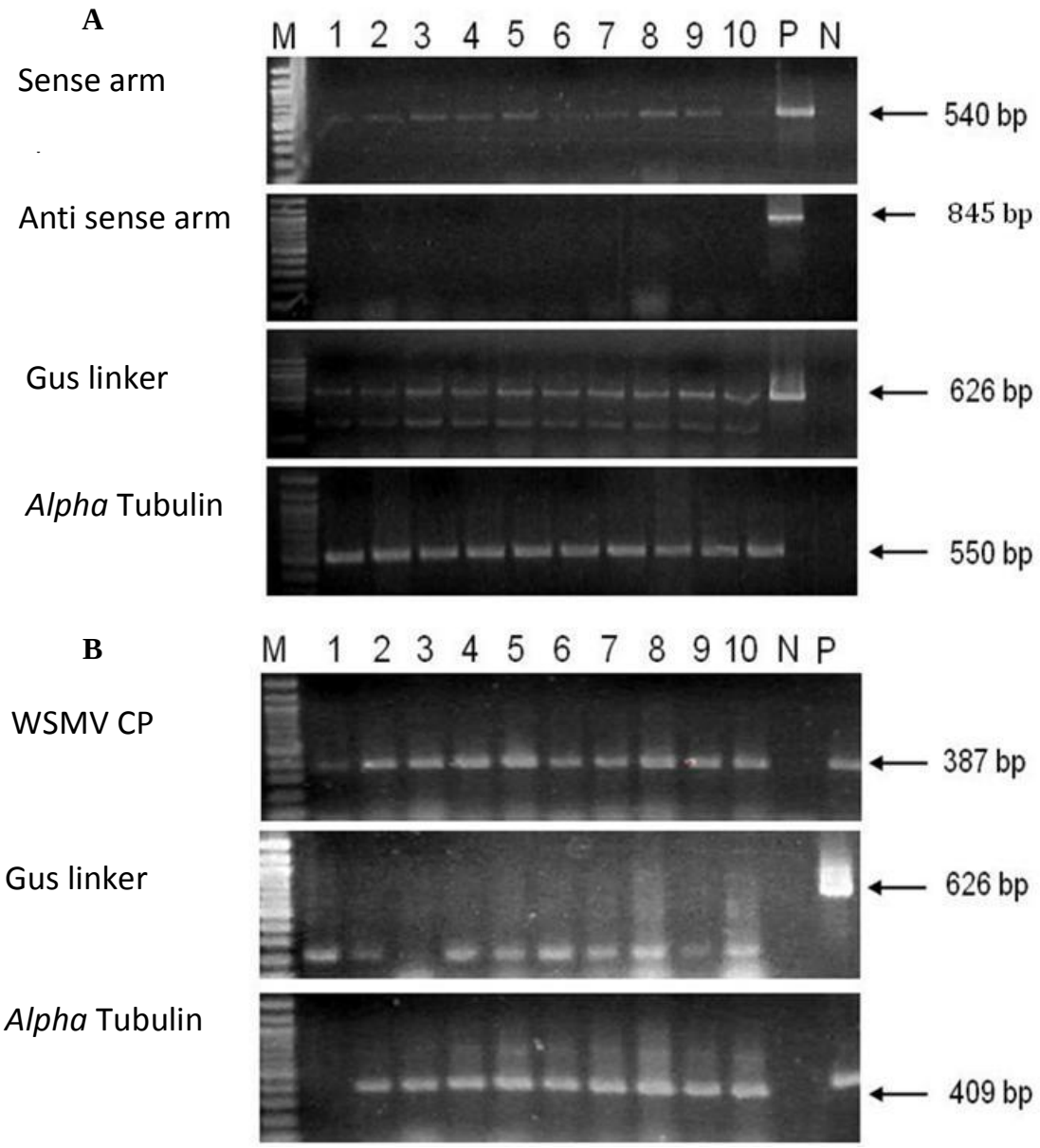

Figure 8. Molecular analyses of the T_2 WSMV CP RNAi transgenic plant line 110-B8. M, 100 bp DNA marker; 1-10, ten T_2 transgenic wheat plants; N, non-transgenic wheat plant; N1, H2O; P, WSMV CP-pANDA mini initial vector (5 pM). (A) PCR for transgene detection. (B) RT-PCR analyses. P, gDNA.

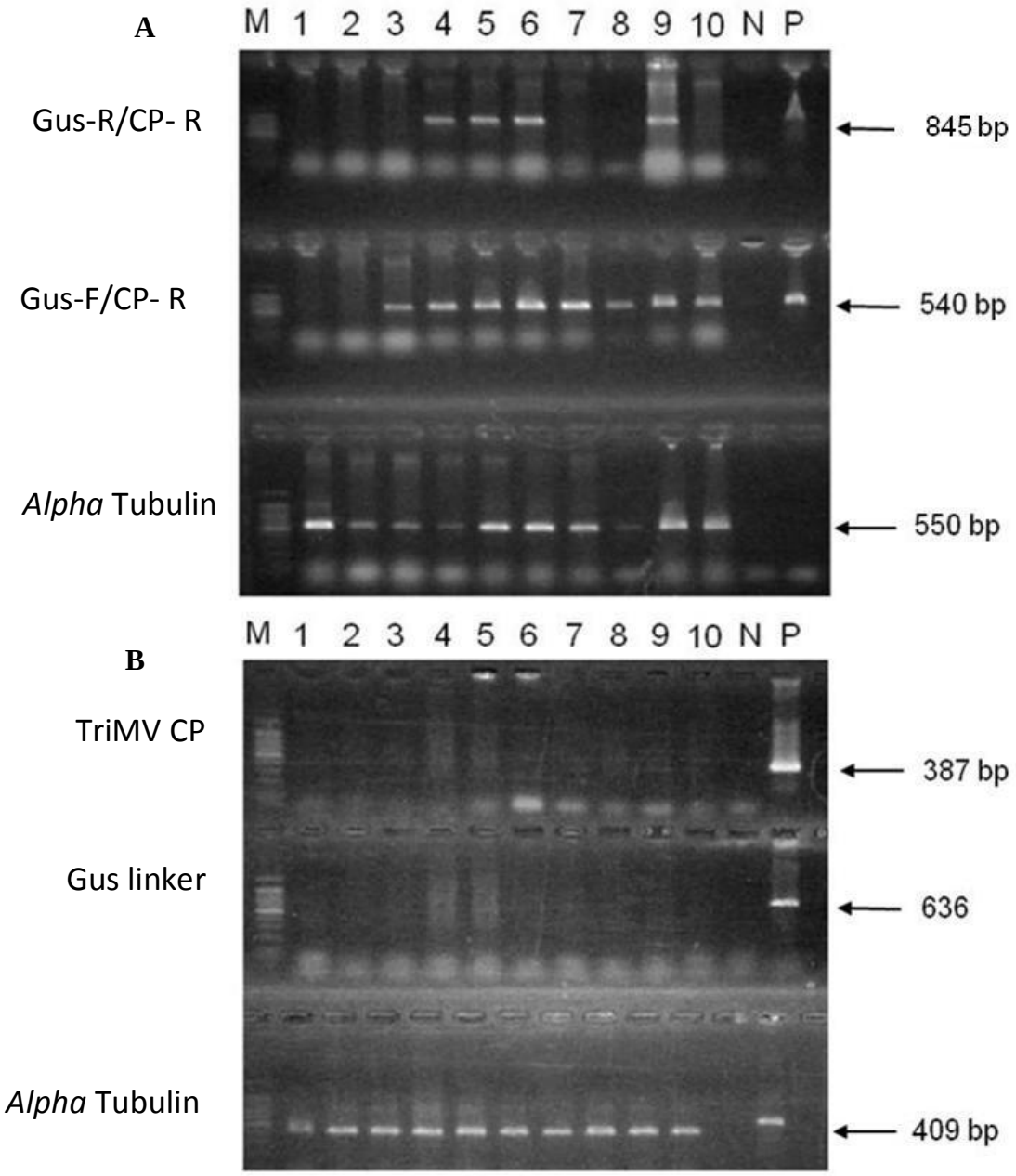

Figure 9. Molecular analyses of the T_2 WSMV CP RNAi line 147- a1. M, 100 bp DNA marker; 1-10, ten T_2 transgenic wheat plants; N, non-transgenic wheat plant; N1, H2O; P, WSMV CP-pANDA mini initial vector (5 pM). (A) PCR for transgene detection. (B) RT-PCR analyses. P, gDNA.

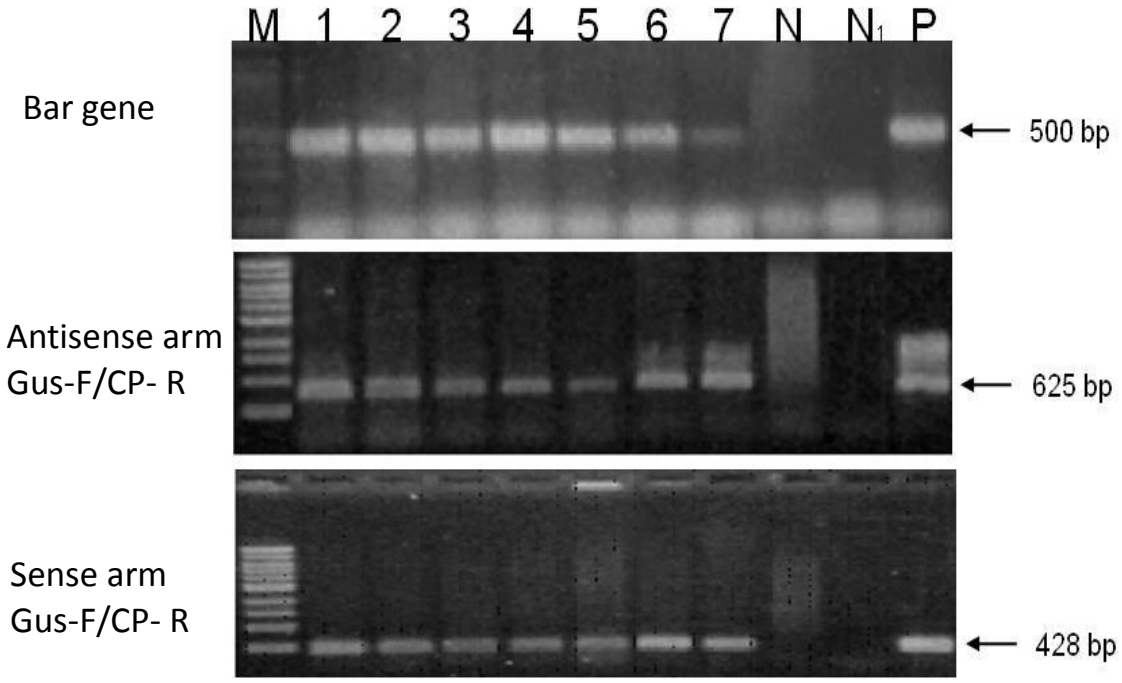

Figure 10. PCR analyses on gDNA of T_0 TriMV CP RNAi transgenic wheat. M, 100 bp DNA marker; 1-8, eight independent transgenic wheat plants; N_1, non-transgenic wheat plant; N_2, H_2O; P, TriMV-pANDA mini (5 pM), bombardment vector**.**

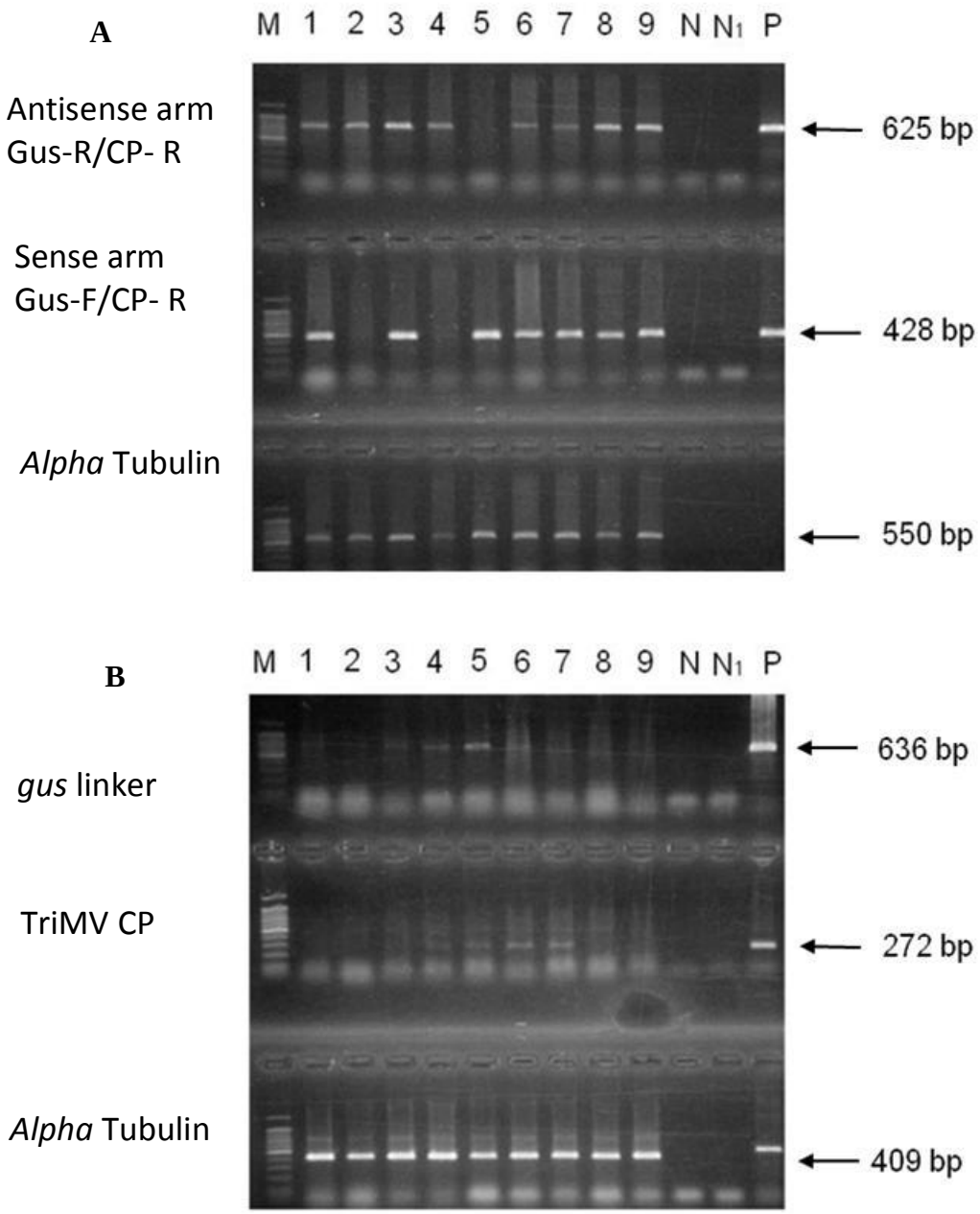

Figure 11. Molecular analyses of the T_2 TriMV CP RNAi transgenic plants line 201- A19. M, 100 bp DNA marker; 1-10, ten T_2 transgenic wheat plants; N, non-transgenic wheat plant; N_1, H_2O; P, TriMV CP-pANDA mini initial vector (5pM). (A) PCR for transgene detection. (B) RT-PCR analyses. P, gDNA.

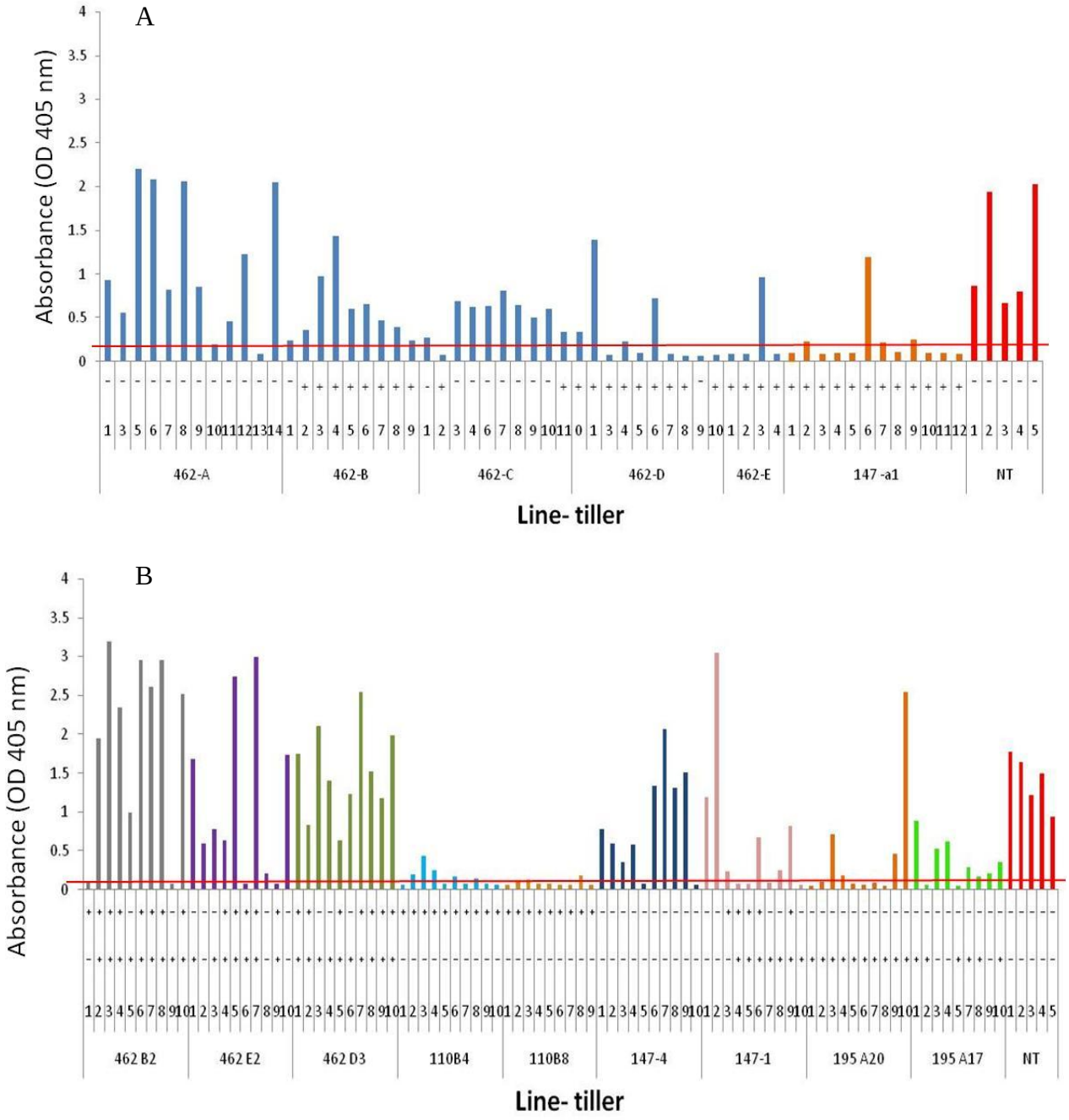

Figure 12. ELISA results T_1 (A) and T_2 (B) generation WSMV CP hairpin construct. Axis Y virus titer, axis X presence or absence of hairpin transgene (plus & minus symbols) in the plants evaluated in the tiller non-transgenic control plants (NT). The red line represents the threshold between positive (above) and negative (below) virus presence.

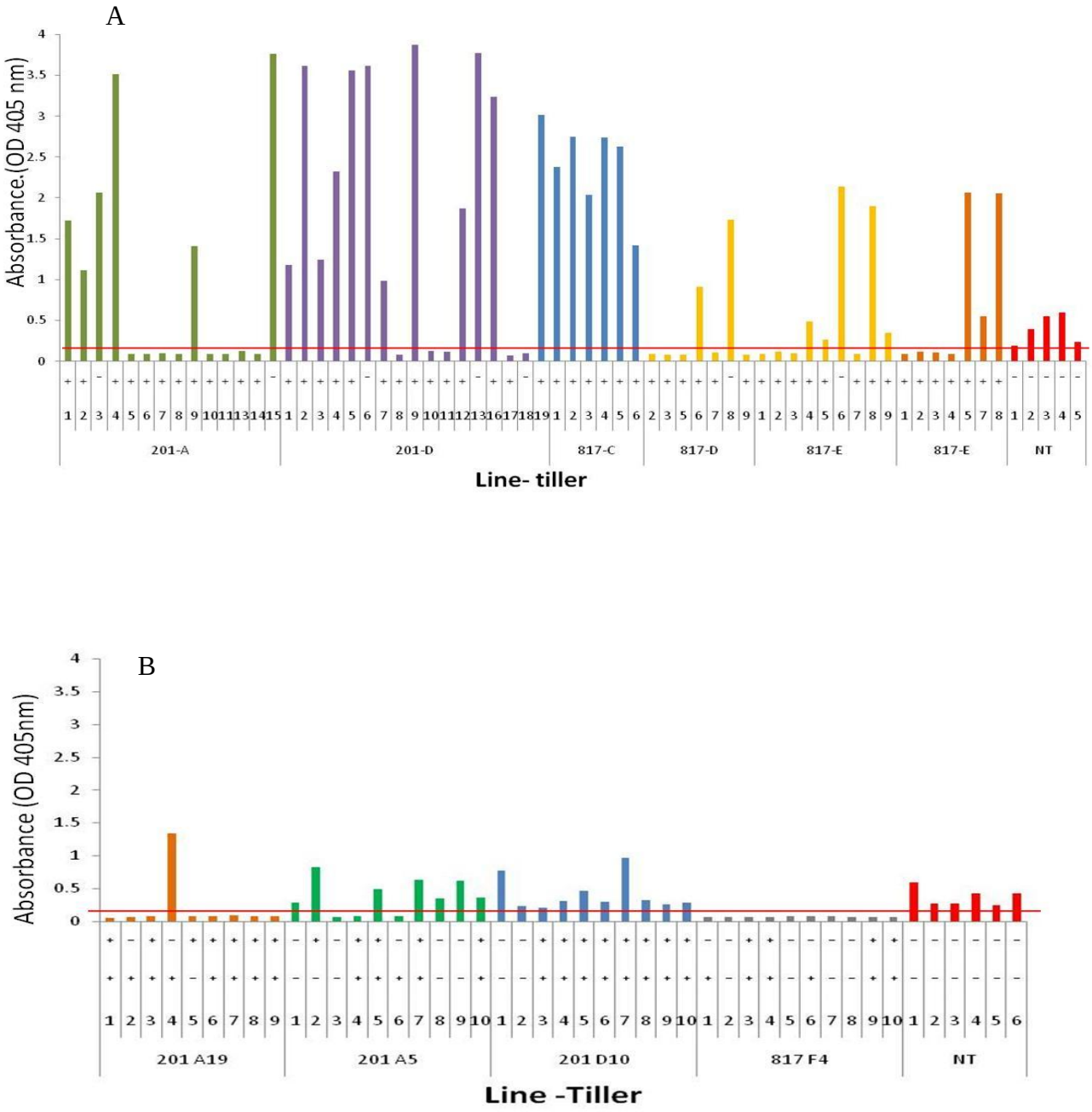

Figure 13. ELISA results T_1 (A) and T_2 (B) generation TriMV CP hairpin construct. Axis Y virus titer, axis X presence or absence of hairpin transgene (plus & minus symbols) in the plants evaluated in the tiller non-transgenic control plants (NT). The red line represents the threshold between positive (above) and negative (below) virus presence.

References And/Or Bibliography

Anandalakshmi R., Pruss G., Ge X., Marathe R., Mallory A., Smith., Vance V., 1998. A viral suppressor of gene silencing in plants. PNAS 95, 13079-13084.

Baulcombe D., 2004.RNA silencing in plants. Nature 431, 356-363.

Beclin C., Boutet S, Waterhouse P., Vaucheret H., 2002. A branched pathway for transgene-induced RNA silencing in plants. Current Biology 12,684–688.

Blanc, S., Lopez-Moya, J.J., Wang, R.Y., Garcia-Lampasona, S., Thornbury, D.W., Pirone, T.P.,1997. A specific interaction between the coat proteinand helper component correlates with aphid transmission of a potyvirus. Virology 231, 141–147.

Blanc S., Ammar E.D., Garcia-Lampasona S., Dolja V.V., Llave C., Baker, J., Pirone T.P., 1998. Mutations in the potyvirus helper component protein: effects on interaction with virions and aphid stylets. Journal of General Virolology 79, 3119–3122.

Beachy R.N., Loesch-Fries S, Tumer N.E., 1990. Coat Protein –mediate resistance against virus infection. Annual Review Phytipathology 28, 451-474.

Bernstein, E., A. A. Caudy, S. M. Hammond, and G. J. Hannon., 2001. Role for a bidentate ribonuclease in the initiation step of RNA interference. Nature 409, 363-366.

Bucher E., Lohuis D., van Poppel P. M. J. A., Geerts-Dimitriadou C., Goldbach R., Prins M., 2006. Multiple virus resistance at a high frequency using a single transgene construct. Journal of General Virology 87, 3697-3701.

Bockus W. W., Appel J. A., Bowden R.L., Fritz A. K., Gill B. S., Martin T. J., Seifers D. L., Brown-Guedira G. L., Eversmeyer M. G., 2001. Success stories: Breeding for wheat disease resistance in Kansas. Plant Disease 85, 453–461.

Buker S.M., Iida .T, Bühler M., Villén J., Gygi S.P., Nakayama J., Moazed D., 2007. Two different Argonaute complexes are required for siRNA generation and heterochromatin assembly in fission yeast. Nat Struct Mol Biol 14, 200-206.

Carrington J.C., Jensen P.E., Schaad M., 1998 Genetic evidence for an essential role for potyvirus CI protein in cell-to-cell movement. The Plant Journal 14, 393–400.

Christensen, A.H., and Quail, P.H., 1996. Ubiquitin promoter-based vectors for high level expression of selectable and/or screenable marker genes in monocotyledonous plants. Transgenic Res. 5, 213-218.

Cronin S., Verchot J., Haldeman-Cahill R., Schaad M.C., Carrington, J.C., 1995. Long-distance movement factor: a transport function of the potyvirus helper component proteinase. Plant Cell 7, 549–559.

Dangl, J. L., Jones, J. D. G., 2001. Plant pathogens and integrated defence responses to infection. Nature 411, 826–833.

De la Cruz J., Kressler D., and Linder P., 1999. Unwinding RNA in Saccharomyces cerevisiae: DEAD-box proteins and related families. Trends Biochem. Sci. 24, 192–198.

Diaz-Pendon J. A., Truniger V., Nieto C., Garcia-Mas J., Bendahmane A., Aranda M. A., 2004. Advances in understanding recessive resistance to plant viruses. Molecular Plant Pathology 5, 223-233.

Dinant S, Maisonneuve B, Albouy J, et al., 1997. Coat protein gene-mediated protection in *Lactuca sativa* against lettuce mosaic potyvirus strains. Molecular Breeding 3, 75–86.

Dolja V.V., Haldeman R., Robertson N.L., Dougherty W.G., Carrington J.C., 1994. Distinct functions of capsid protein in assembly and movement of tobacco etch potyvirus in plants. EMBO J. 13, 1482–1491.

Duprat A., Caranta C., Revers F., Menand B., Browning K. S., Robaglia C., 2002. The Arabidopsis eukaryotic initiation factor (iso)4E is dispensable for plant growth but required for susceptibility to potyviruses. Plant J 32, 927–934.

Fauquet M., Mayo M.A., Maniloff J., Desselberger U., Ball L.A. (Eds)., 2005. Virus Taxonomy, VIIIth Report of the International Committee on Taxonomy of Viruses".

Farnham, G. and Baulcombe, D.C., 2006 Artificial evolution extends the spectrum of viruses that are targeted by a disease-resistance gene in potato. Proc. Natl Acad. Sci. 103, 18828–18833.

Fladung M., 1999. Gene stability in transgenic aspen (Populus). I. Flanking DNA sequences and T-DNA structure. Molecular Genetics and Genomis 260: 574-581.

Feldmann K. A., Coury D.A., Christianson M.L., 1997. Exceptional segregation of a selectable marker (KanR) in Arabidopsis identifies genes important for gametophytic growth and development. Genetics 147: 1411-1422.

Fellers J., Wan J., Hong Y., Collins G. B., Hunt A. G., 1998. In vitro interactions between a potyvirus-encoded, genomelinked protein and RNA-dependent RNA polymerase. General Virology 79, 2043- 2049.

Fernández A., Guo H. S., Sáenz P., Buela L. S., Gómez M. García J.,1995. The motif V of plum pox potyvirus CI RNA helicase is involved in NTP hydrolysis and is essential for virus RNA replication. Nucleic Acid Research 25, 4474-4480.

Fernandez A., Garcia J. A., 1996. The RNA helicase CI from plum pox potyvirus has two regions in binding to RNA.FEBS Lett. 338, 206 -210.

Fire A., Xu S., Montgomery M.. Kostas S. A., Driver S. E., Mello C., 1998 Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans* .Nature 391, 806-811.

Friebe B., Mukai Y., Dhaliwal H.S. , Martin T. J., Gill B.S., 1991. Identification of alien chromatin specifying resistance to wheat streak mosaic and greenbug in wheat germplasm by C-bandingand in situ hybridization. Theor. Appl. Genet. 81, 381-389.

Friebe B., Qi L. L., Wilson D. L., Chang Z. J., Seifers D. L., Martin T. J., Fritz A. K., Gill B. S., 2009. Wheat–Thinopyrum Intermedium Recombinants Resistant to Wheat Streak Mosaic Virus and Triticum Mosaic Virus. Crop science 49, 1221-1226

Fu D., Uauy C., Blechl A., Dubcovsky J., 2007 RNA interference for wheat functional gene analysis. Transgenic Research 16, 689–701.

Gill Gura T., 2000. A silence that speaks volumes. Nature 404, 804-808.

Haley, S.D., Martin T.J.,Quick J.S. Seifers, D.L., Stromberger J.A., Clayshulte S.R., Clifford B.L., Peairs F.B., Rudolph J.B., Johnson J.J., Gill B.S., Griebe B., 2002. Registration of

CO960293-2 wheat germplasm resistant to Wheat streak mosaic virus and Russian wheat aphid. Crop Sciences 42,1381–1382.

Hall T. M. Structure and Function of Argonaute Proteins. Structure.2005. 13,1403-1048.

Hamilton, A. J., and D. C. Baulcombe., 1999. A species of small antisense RNA in posttranscriptional gene silencing in plants. Science 286,950-952.

Hammond, S. M., E. Berstein, D. Beach, and G. J. Hannon., 2000. An RNA-directed nuclease mediates post-transcriptional gene silencing in *Drosophila* cells. Nature 404, 293-296.

Hammond S. M., Caudy A. A., Hannon G. J., 2001. Post-transcriptional gene silencing by doble stranded RNA. Nature 2,110-119.

Helliwell C., Waterhouse P., 2005. Constructs and methods for hairpin RNA-mediated gene silencing in plants. Methods in Enzymology 392, 24-35.

Hily J. M., Scorza R., Webb K., Ravelonandro M., 2005. Accumulation of the Long Class of siRNA Is Associated with Resistance to Plum pox virus in a Transgenic Woody Perennial Plum Tree. MPMI 18, 794–799.

Hull, R., 2002. Matthews' Plant Virology, 4th edn. San Diego:Academic Press.

Jorgensen R., 2002. RNA traffics information systemically in plants. Proc Natl Acad Sci. 99, 11561-11563.

Léonard S., Plante D., Wittman S., Daigneault N., Fortin,M.G., Laliberté, J.-F., 2000. Complex formation between potyvirus VPg and translation eukaryotic initiation factor 4E correlates with virus infectivity. J. Virol. 74, 7730–7737.

Li J., Zhao W., Li Q., ye X., An B., Li X., Zhang X., 2005. RNA silencing of *waxy* gene result in low levels of amylase in the seeds of transgenic wheat (*Triticum aestivum* L.). Acta Genetica Sinica 32, 846-854.

Li Z., Liu Y., Berger P. H., 2005. Transgenic silencing in wheat transformed with the WSMV-CP gene. Biotechnology 4, 62-68.

Lindbo J.A., Dougherty W.G., 1992. Untranslatable transcripts of the tobacco etch virus coat protein gene sequence can interfere with tobacco etch virus replication in transgenic plants and protoplasts. Virology 189, 725-733.

Lindbo J. A., Silva-Rosales L., Proebsting W. M., and Dougherty W. G., 1993. Induction of a Highly Specific Antiviral State in Transgenic Plants: Implications for Regulation of Gene Expression and Virus Resistance. Plant Cell 5, 1749-1759.

MacDiarmid R., 2005. RNA silencing in productive virus infections. Annual Review of Phytopathology 43, 523-544.

Maia I. G., Haenni A. and Bernardi F., 1996. Potyviral HC-Pro: a multifunctional protein. Journal of General Virology 77, 1335-1341.

Maule A. J. , Caranta C., Boulton M. I., 2007. Sources of natural resistance to plant viruses: status and prospects. Molecular Plant Pathology 8, 223-231 .

Meister G., Tuschl T., 2004. Mechanisms of gene silencing by double-stranded RNA. Nature 431, 343-349.

Mérai Z., Kerényi Z., Kertész S, Magna M., Lakatos L., Silhavy D., 2006. Double-Stranded RNA Binding May Be a General Plant RNA Viral Strategy To Suppress RNA Silencing. Journal of Virology 80, 5747–5756.

Melo C. C., Conte D., 2004. Revealing the world of RNA interference. Nature 431, 338-342.

Miki D., Shimamoto K., 2004. Simple RNAi vectors for stable and transient suppression of gene function in rice. Plant Cell Physiology 45, 490–495.

Napoli C.,' Lemieux C., Jorgensen R., 1990. lntroduction of a Chimeric Chalcone Synthase Gene into Petunia Results in Reversible Co-Suppression of Homologous Genes Ín trans .The Plant Cell 2, 279-289.

Nicaisea V., Galloisb J., Chafiaia F., Allenc L. M., Schurdi-Levrauda V., Browningc K.S., Candressea T., Carantab C., Le Galla O., German-Retana S., 2007. Coordinated and selective recruitment of eIF4E and eIF4G factors for potyvirus infection in Arabidopsis thaliana FEBS Letters 581, 1041- 1046.

Niu QW, Lin SS, Reyes JL, Chen KC, Wu HW, Yeh SD, Chua NH., 2006. Expression of artificial microRNAs in transgenic Arabidopsis thaliana confers virus resistance. Nature Biotechnology 24, 1420-1428.

Parker L., Kendall A., Berger P.H., Shiel P.J., Stubbs G., 2005. Wheat streak mosaic virus Structural parameters for a Potyvirus. Virology 340, 64 – 69

Pooggin M, Dreyfus M., Hohn T., 2001. mRNA Enigmas: The silence of the genes. Int Arch Biosc 1023 -1026.

Riechmann J. L., Lain S., Garcia J. A., 1992. Highlights and prospects of potyvirus molecular biology. Journal of General Virology 73- 1-16.

Risseeuw E., Franke-van Dijk M. E., Hooykaas P. J., 1997. Gene targeting and instability of Agrobacterium T-DNA loci in the plant genome. Plant Journal 11, 717-728.

Romano, N., Macino, G., 1992. Quelling: transient inactivation of gene expression in Neurospora crassa by transformation with homologous sequences. Mol. Microbiol. 6, 3343–3353.

Romano E., Soares A., Proite K., Neiva S., Grossi M., Faria J. C., Rech E. L. , Aragão F., 2005. Transgene elimination in genetically modified dry bean and soybean lines. Genetic and Molecular Research 4, 177-184.

Ruiz V., and Olivier V., 2007. Viral suppression of RNA silencing: 2b wins the Golden Fleece by defeating Argonaute. BioEssays 29, 319-323.

Sambrook J., Rusell D. W., 2001. Molecular Cloning: A Laboratory Manual. 3th ed, NY: Cold Spring Harbor Laboratory Press

Sato M., Nakahara K., Yoshii M., Ishikawa M., Uyeda I., 2005. Selective involvement of members of the eukaryotic initiation factor 4E family in the infection of *Arabidopsis thaliana* by potyviruses. FEBS Lett. 579, 1167-1171.

Schaad, M. C., P. E. Jensen, and J. C. Carrington., 1997. Formation of plant RNA virus replication complexes on membranes: role of an endoplasmic reticulum-targeted viral protein. EMBO J. 16, 4049-4059.

Seifers D. L., Martin T.J., Harvey T. L., Stack J.P., Ryba-White M., Harber S., Krikhin O., Spicer V., Lovat N., Yamchuk A., StandingK. G., 2007. Triticum mosaic virus: A New Virus Isolated From Wheat In Kansas. Plant Disease 92, 808-817.

Seifers, D.L., Martin T.J., Harvey T.L., Haber S., 2007. Temperature- sensitive Wheat streak mosaic virus resistance Identified in KS03HW12 wheat. Plant Dis. 91, 1029-1033.

Sivamani E., Brey C. W., Dyer W. E., Talbert L. E., Qu R., 2000. Resistance to wheat streak mosaic virus in transgenic wheat expressing the viral replicase (NIb) gene. Molecular Breeding 6, 469-477.

Sivamani E., Brey C. W., Dyer W. E., Talbert L. E., Qu R., 2002 Resistance to wheat streak mosaic virus in transgenic wheat engineered with the viral coat protein Transgenic Research 11, 31-41.

Srivastava V., Vasil V., Vasil I. K., 1996. Molecular characterization of the fate of transgenes in transformed wheat (*Triticum aestivum* L.). Theoretical and Applied Genetics 92, 834-842.

Slykhuis, J. T., 1955. *Aceria tulipae* Keifer (*Acarina: Eriophyidae*) in relation to spread of wheat streak mosaic virus. Phytopathology 45, 116-128.

Smith N. A., Singh S. P., Wang M., Stoutjesdijk P. A., Green A.G ,. Waterhouse P. M., 2000. Gene expression: Total silencing by intron-spliced hairpin RNAs. Nature 407, 319-320.

Soosaar J.L.M., Burch-Smith T., Dinesh-Kumar S.P., 2005. Mechanisms of plant resistance to viruses. Nature Reviews Microbiology 3, 789-798.

Stanford J.C., Johnston S.A., 1985. The concept of parasite resistance genes from parasites own genome. J.Theor. Biol. 113, 395-405.

Stenger C. , Hein G. L., Gildow F E., Horken K. M., French R., 2005. Plant Virus HC-Pro Is a Determinant of Eriophyid Mite Transmission. Journal of virology 79, 9054–9061.

Stenger C., French R., Gildow F., 2005 Complete Deletion of *Wheat Streak Mosaic Virus* HC-Pro: a Null Mutant Is Viable for Systemic Infection Journal of Virology 79, 12077–12080.

Stenger DC, Young BA, French R. 2006. Random mutagenesis of Wheat streak mosaic virus HC-Pro: noninfectious interfering mutations in a gene dispensable for systemic infection of plants. Journal of General Virology 87, 2741–2747.

Stenger, D. C., Hein, G. L. & French, R. 2006. Nested deletion analysis of Wheat streak mosaic virus HC-Pro: mapping of domains affecting polyprotein processing and eriophyid mite transmission. Virology 350, 465–474.

Tennant P., Fermin G., Fitch M. M., Manshardt R. M., Slightom J. L., Gonsalves D., 2001. Papaya ringspot virus resistance of transgenic Rainbow and SunUp is affected by gene dosage, plant development and coat protein homology. European Journal of Plant Pathology 107, 645–653.

Tenger, D.C., Hall, J.S., Choi, I.-R., French, R., 1998. Phylogenetic relationships within the family Potyviridae: wheat streak mosaic virus and brome streak mosaic virus are not members of the genus Rymovirus. Phytopathology 88, 782– 787.

Thivierge K., Nicaise V., Dufresne P., Cotton S., Laliberté J.F., Le Gall O., Fortin M. G., 2005.Plant Virus RNAs. Coordinated Recruitment of Conserved Host Functions by (+) ssRNA Viruses during early Infection Events. Plant Physiology 138, 1822-1827.

Urcuqui-Inchima S., Haenni A. L., Bernardi F. 2001. Potyvirus proteins: a wealth of functions. Virus research 74, 157-175.

Seifers D. L., Martin T. J., Harvey T. L., Haber S., Haley S. D. 2006. Temperature Sensitivity and Efficacy of Wheat streak mosaic virus Resistance Derived from CO960293 Wheat. Plant disease 90, 623-628.

Tanzer M. M., Thompson W. F., Law M.D., Wernsman E.A., Uknes S., 1997. Characterization of Post-Transcriptionally Suppressed Transgene Expression That Confers Resistance to Tobacco Etch Virus Infection in Tobacco. Plant Cell 9, 1411–142.

Yan K. S., Yan S, Farooq A., Han A., Zeng L., Zhou M. 2003 Structure and conserved RNA binding of the PAZ domain. Nature 426, 468-474.

Zar, J. H. 1999. Biostatistical Analysis, 4th ed. Prentice Hall, Upper Saddle River, NJ