VIRUS-INDUCED GENE SILENCING OF PUTATIVE *DIURAPHIS NOXIA* (KURDJUMOV) RESISTANCE GENES IN WHEAT

by

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Abstract

The Russian wheat aphid *Diuraphis noxia* (Kurdjumov) is a serious pest of world cereal grain crops, primarily barley and wheat. A phenotypic characteristic of *D. noxia* feeding, leaf rolling, creates a leaf pseudo gall which protects aphids, making it difficult to treat infested plants with insecticides or biological control agents. Therefore, the use of *D. noxia*-resistant crops is a desirable aphid management tactic. Because of the development of virulent *D. noxia* biotypes, the identification of new sources of barley and wheat resistance is necessary. Virus-induced gene silencing (VIGS) utilizes the plant defense system to silence viruses in inoculated plants. The accumulation of virus RNA in plants triggers the defense system to silence sequences homologous to the introduced virus and sequences of interest from a plant are inserted into the virus and silenced along with the virus. The VIGS method was tested to determine the ability of barley stripe mosaic virus (BSMV) to serve as a VIGS vector in wheat plants containing the *Dnx* gene for resistance to *D. noxia*. *Dnx* leaves with silenced BSMV virus yielded *D. noxia* populations that were significantly no different from populations produced on healthy *Dnx* leaves. Thus, BSMV silencing does not interfere with *Dnx* resistance. Several different methods were examined to determine how best to confine aphids to the silenced leaf, and a modified plastic straw cage was chosen as the optimum cage type. Microarray and gene expression data were analyzed to select two NBS-LRR type disease resistance protein genes - TaAffx.104814.1.S1_at and TaAffx.28897.1.S1 - (NBS-LRR1 and NBSLRR2), in order to assess their role in *Dnx* resistance. NBS-LRR1 and NBSLRR2 were silenced by inoculating leaves of *Dnx* plants with barley stripe mosaic virus (BSMV) containing sequences of each gene. Controls included *Dnx* and Dn0 plants inoculated with BSMV and non-BSMV inoculated plants. Aphids were allowed to feed on control and treatment plants to assess aphid population and

mean weight of aphids surviving at the end of the experiment. There were no differences among treatments based on aphid population, but there were significant differences the mean weights of aphids reared on several different treatments.

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CHAPTER 1 - Introduction and Literature Review

Russian Wheat Aphid History and Biology

The Russian wheat aphid, *Diuraphis noxia* has a characteristic green body color, reduced cornicles, and a caudate tail projection. The aphid is native to Iran, Afghanistan, southern Russia, and other countries along the Mediterranean Sea (Hewitt et al. 1984), and is a major pest of wheat and barley in most cereal growing regions of the world except for Australia (Quisenberry and Peairs 1998). Aphids cause severe damage to plants, and symptoms include prevention of leaf unrolling, leaf chlorosis, and plant death (Walters et al. 1980). As a result, damaged wheat may be unmarketable. *D. noxia* damage can cause the cereal industry several hundred million dollars of losses every year (Webster et al. 2000).

Different management tactics have been employed to combat aphid attack including the deployment of biocontrol agents (Prinsloo 2000, Wraight et al. 1993) and insecticides (Webster et al. 1987) have been used to prevent *D. noxia* outbreaks. Components of the *D. noxia* life cycle, such as the tendency to seek shelter in rolled leaves, make it difficult to deploy insecticides and biocontrol agents.

D. noxia has developed biotypes capable of overcoming previously *D. noxia* resistant wheat lines (Puterka et al. 1992, Basky 2003, Hawley et al. 2003, Smith et al. 2004, Tolmay et al. 2007). For this reason, it is imperative to identify wheat lines that are naturally resistant to aphid attack. North American biotype 2 (RWA2) was first described in Colorado in 2003 (Haley et. 2004). Thus far only the *Dn7* resistance gene from rye confers resistance to RWA2 (Burd et al. 2006), although the *Dnx* gene has been shown to express partial resistance to RWA2 (Lazzari et al. 2009) and full resistance to RWA1 (Liu et al. 2005). *Dnx* plants also exhibit a

hypersensitive reaction at aphid feeding sites and the *Dnx* gene is inherited in a semi-dominant manner (Liu et al. 2001, Haley et al. 2004, Smith et al. 2004), suggesting that the relationship of *Triticum aestivum* with *D. noxia* may be a gene-for-gene interaction.

Painter (1951) described resistant cultivars as those which contain "heritable characteristics…which influence the ultimate degree of damage done by the insect". The resistant plants exhibit better overall survival and yield than plants that are susceptible (Smith 1989). Resistance can be separated into several categories. The antibiosis category of resistance affects the pest's ability to feed and survive on the host plant due to plants factors limiting insect survival and reproduction. Insect pest behavior may be altered in other forms of resistance. Pests may be able to feed on a particular variety, but with less success than other varieties and thus prefer other hosts. This is the case with plants that possess antixenosis resistance. The third category of resistance is termed tolerance, and is described by the plant's ability to tolerate insect attack. Tolerance is measured as the plants ability to survive and thrive in spite of insect infestation. Because tolerance does not exert selection pressure on insect populations, it may be less likely to trigger the development of virulent biotypes compared to antibiosis resistance.

Previous results have indicated allelic or linked *D. noxia* resistance genes are located on the short arm of wheat chromosome 7DS (Liu et al. 2005) and are designated as *Dn1, Dn2, Dn5, Dn6,* and *Dnx*. The *Dn4* gene is located on the short arm of wheat chromosome 1DS as is the *Dn7* gene, a *1RS/1BL* translocation (Liu et al. 2001, 2002). Chinese Spring wheat lines have been created to contain deletions of particular stretches of the chromosome (known as bins) or the arm or the entire chromosome. These deletion lines may be used as templates in PCR reactions to map the specific location of candidate EST sequences.

There are five classes of constitutively expressed plant resistance genes. These include four classes that have similar overall structure to the NBS-LRR genes with some variations. Class I, II, and III resistance genes lack transmembrane domains and may have little involvement with other gene products outside the cell (Smith 2005). Several Class II genes are involved with plant resistance response, especially to arthropods. In particular, *Mi-1.2* plays a role in resistance to arthropods and nematodes. The *Mi-1.2* gene functions to confer resistance to the *Meloidogyne* species of nematode (Milligan et al. 1998) and whiteflies (Nombela et al. 2000, 2001) and also to the potato aphid, *Macrosiphum euphorbiae* Thomas (Rossi et al. 1998). Class II includes the actual NBS-LRR genes that have a structure of leucine rich repeats (LRR) and nucleotide binding sites (NBS) in addition to either a leucine zipper (LZ) or coiled coil region (CC). Class III resistance genes encode for a Toll and Interleukin 1 region instead of the CC region while Class IV and Class V genes have extracellular LRR regions. Class V is also involved in serine/threonine kinase coding.

There are several key signaling pathways involved in plant defense responses to attack by viruses, arthropods, nematodes, or fungi. The jasmonate pathway is of particular interest because production of methyl jasmonate has been linked to plant defensive responses to several pathogens and arthropods. Two-spotted spider mites are more virulent on tomato plants that exhibit decreased levels of jasmonic acid (JA) (Li et al. 2002). Ellis et al. (2002) documented the role of JA in resistance to a plant fungus, *Erysiphe cichoracearum* and the green peach aphid, *Myzus persicae* Sulzer.. Several products from the JA pathway, such as *FAD3C, DAD1 LOX1, ACSI, 12-OPR,* OPDA hydrolases, and 12-OPDA ABC transporters were found to upregulated in resistant *Dnx* plants versus the susceptible *Dn0* plants (Smith et al. 2010). Birkett et al. (1999) found that wheat plants treated with the volatile cis-jasmone were less

attractive to cereal aphids but also contained higher levels of aphid predators. These results indicate that jasmonate volatiles may deter herbivorous insects but also recruit predators.

Virus-Induced Gene Silencing

Reverse genetics may be one path to discover genetic components of plant resistance. Reverse genetics allows the investigator to silence a gene and then observe the effect of that gene. It is a quicker method than more traditional approaches, such as the creation of transgenic plants. The use of Virus-Induced Gene Silencing (VIGS) is one such method available to scientists. VIGS uses the plant's natural defense system to detect and destroy high levels of double stranded RNA. Any sequences homologous to the dsRNA are also targeted and destroyed. In viral inoculation, this results in some symptom-free leaves. Kumagai et al. (1995) used the tobamovirus to silence the phytoene desaturase gene (PDS) in *Nicotiana benthamiana* to demonstrate that knockdown of PDS influences the carotenoid pathway. In addition, the phenotype resulting from PDS silencing has been used as a visual marker in other VIGS experiments utilizing other viral vectors and host plants (Scofield et al. 2005, Brunn-Rasmussen et al. 2007).

A plant gene sequence may also be inserted into the viral construct so that the VIGS process will silence both the virus and the insert-targeted gene. If the gene is linked to resistance, then the plant with the silenced gene will show more susceptibility than control plants. This method of gene screening is more rapid than the traditional creation of transgenic plants. Another benefit of VIGS is that the process does not exhibit systemic effects throughout the plant. Inoculation of the second leaf of a 10 day-old wheat seedling results in virus symptom expression on the second and third leaves, while the fourth leaf emerges without symptoms, demonstrating the silencing effect (Scofield et al. 2005). The localized response allows the

researcher to examine the effect of silencing of genes that are key to early development, but to do so in mature tissue that will not have a lethal effect from the silencing.

Scholthof et al. (1996) utilized the VIGS method to insert foreign genes into virus vectors and observed successful expression within the virus. Various factors, such as choice of viral vector, the type of host plant, growth conditions, and size of a gene insert, can influence the success of a VIGS application. The size of the gene insert can greatly impact the efficiency of VIGS-induced gene silencing. Bruun-Rasmussen et al. (2007) demonstrated more likely for VIGS to be successful when inserts ranging from 128 nt to 584 nt are used, typically with gene knockdown rates of 70-84%, respectively. Scofield et al. (2005) used Barley Stripe Mosaic Virus (BSMV) to examine the role of the *Lr*21 leaf rust resistance gene in wheat and found the system to be viable with an insert size of 120 nt.

The VIGS process may be used in a variety of plant species including dicots and more recently, grass species. Early VIGS work focused on silencing in dicot species such as tomato *Solanum lycopersicum* (Liu et al. 2002), potato *Solanum tuberosum* (Brigneti et al. 2004), and Arabidopsis *Arabidopsis thaliana* (Burch-Smith et al. 2006). Holzberg et al. (2002) demonstrated the first case of VIGS silencing in a monocot, using barley stripe mosaic virus (BSMV) to silence PDS and GFP genes in barley. Previously, VIGS has been used to screen barley genes for their role in pathogen resistance. Hein et al. (2005) used BSMV to knockout *Hsp90*, a gene involved in the *Mla13* response to *Blumeria graminis* f. sp. *hordei,* and to verify its role in plant response to pathogen attack. Scofield et al. (2005) used BSMV to study *Lr21* resistance to wheat leaf rust.

Several plant viruses have been used as VIGS vectors, including *Tobacco mosaic virus* (Kumagi et al. 1995), *Potato virus X* (Ratcliff et al. 1997), and *Tobacco rattle virus* (Ratcliff et

al. 2001). There are several viral properties to consider when choosing an appropriate VIGS vector. Some viruses, such as *Tobacco etch virus* (TEV) can generate mechanisms to overcome gene silencing in the plant and are not suitable for VIGS (Anandalakshmi et al. 1998). Most VIGS vectors are single-stranded RNA viruses due to the fact that the closed capsid structure of DNA viruses can hinder the size of gene insert allowed.

VIGS offers several advantages over other methods of gene analysis. Virus-based experiments are more rapid than creating plant mutants and growing out seedlings to observe the effect of a particular silenced gene. VIGS experiments can also be carried on mature plants and allow for the silencing of genes or pathways that might be lethal in young plants when silenced by transformation.

Due to the ability of *D. noxia* to overcome plant resistance and develop virulent biotypes, it is imperative to identify new sources of plant resistance. *Dnx* resistance has been mapped to chromosome 7DS, but the exact gene has not been identified or cloned. My objective was to use VIGS as a rapid method to screen candidate wheat ESTs for their role in *D. noxia* resistance. Four candidates were chosen because preliminary microarray data indicates that their level of constitutive expression is greatly elevated in wheat plants containing *Dnx* compared to susceptible plants lacking *Dnx* (Table 1.1). The genes included two different NBS-LRR genes, an allene oxide cyclase gene from the jasmonic acid (JA) pathway that is highly activated in *D. noxia* biotype 1 resistant plants (Liu et al. unpubl.), and a gene for isoflavone reductase, a key downstream defense response gene, which has been mapped to the bin on wheat 7DS containing *Dnx* (Hossain et al. 2005) (Table 1.2). If *Dnx* or genes related to it can be identified, they may be cloned and inserted into *D. noxia*-susceptible wheat varieties.

Hypothesis and Objectives

Dnx resistance results in decreased *D. noxia* survival and fecundity in resistant plants compared to susceptible plants. Therefore, silencing *Dnx* should result in a susceptible plant phenotype that allows greater *D. noxia* population increases compared to resistant control plants. My hypothesis was that *D. noxia* population growth could be used to verify the role of candidate wheat genes in *D. noxia* resistance. The objectives of my research were:

1. To use bioinformatics and data mining to identify genes that could play a role in wheat resistance to *D. noxia* biotype 1
2. To evaluate BSMV as a VIGS vector in *Dnx* wheat and *D. noxia* interactions.
3. To use VIGS to silence candidate genes in *Dnx* wheat and assess the silencing effect on *D. noxia* resistance.

Materials and Methods

VIGS Feasibility Study

VIGS was used to silence the wheat defensin gene (NCBI accession: AB089942, Affymetrix: Ta. 14281.1.S1_at) to test the effect of silencing on resistance to *D. noxia* biotype 1. Six resistant plants each of *Dnx* (resistant) and KS92 or Jagger (susceptible) plants grown in the greenhouse (14:10 light:dark, 24/20°C day/night) were infected with Barley Stripe Mosaic Virus (BSMV) with the defensin gene silenced. Viral constructs, inoculum, and methods of silencing were those of Scofield et al. (2005). A second treatment consisted of plants infected with BSMV only (no silenced gene) to insure that the gene and not the virus was causing any observed effects. A third treatment consisted of resistant and susceptible plants with no virus. All plants were infested five, fourth-instar *D. noxia* biotype 1. At 7 days post-infestation, the numbers of aphids on each plant, plant chlorosis, and plant leaf rolling symptoms were recorded. At 14 days post-infestation, additional plants with the silenced defensin gene and virus control plants were scored to determine the effect of a longer post-infestation period on symptom expression. All data were then analyzed using the SAS Proc GLM procedure (SAS Institute 1985). Differences between treatment means were separated using the Duncan test at $\alpha = 0.05$.

To determine the feasibility of confining aphids to a silenced leaf, the preliminary experiment was repeated with the following conditions and modifications. 20 *Dnx* plants and 20 Jagger plants were placed in separate growth chambers (25°C night, 20°C day, 14:10 light/dark photophase). The third leaves of plants were inoculated with BSMV RNA and allowed to

develop viral symptoms and for silencing to become effective. Silenced leaves were visibly greener and healthier than symptomatic leaves. Because silenced leaves were visibly greener and less mottled than symptomatic leaves prior to infestation with RWA, chlorophyll (A + B) content of symptomatic leaves and silenced leaves was measured using a SPAD 502-chlorophyll meter (Minolta Corp.). BSMV RNA was prepared using the protocol of Scofield et al. (2005). Symptomatic leaves were trimmed from the plants, leaving only the healthy silenced leaf. Six *Dnx* plants and six Jagger plants were inoculated with BSMV while six plants of each variety served as non-virus controls. Once the silenced leaf emerged in the BSMV-treated plants (in this case, the fifth leaf) all plants (including the corresponding no virus controls) were infested with 10 late instar *D. noxia* biotype 1 nymphs. Non-virus control plants were infested simultaneously, and all plants were separated in the growth chamber by cloth mesh netting. Aphid numbers, chlorosis, and leaf rolling were recorded at 7 days post-infestation. Data were analyzed using the SAS Proc GLM procedure (SAS Institute 1985). Differences between treatment means were separated usingLSD tests and tested for significance at $\alpha = 0.05$.

D. noxia Single Leaf Cage Type Study

In the VIGS feasibility experiments, *D. noxia* were allowed to move freely over the plant, and could spend time feeding on symptomatic leaves as well as on the silenced leaf. To improve the accuracy of assessing plant phenotypic reactions to *D. noxia* feeding, an experiment was conducted to test different methods of caging aphids on a single wheat plant leaf. Single leaves of 20 day old, non-inoculated Jagger (susceptible) plants were evaluated in each of four different types of cages. There two replicates of each cage type. Aphids were allowed to feed for 18 days on each different cage type.

Earlier VIGS experiments had allowed aphids to feed on the entire plant but this design was not desirable because aphids were collected found on other leaves along with the silenced leaf and it was difficult to determine the effect of silencing on aphid numbers. A modification of the previous experiment involved caging the entire infested VIGS plant but clipping off all leaves except for the silenced leaf. In this way the aphids could be confined to the silenced leaf and observed. However, the removal of leaves could result in additional stress on the plant and this in turn could affect aphid populations. Four methods were tested for their ability to confine aphids to a single leaf with a minimum stress: plastic straw punctured with holes and sealed at end with cotton, paper sleeve punctured with holes and sealed at the base of the leaf with a paper clip, sticky trap (2.5 x 1.3 cm sticky trap, TM company), and clipped leaf design. The straw, sleeve, and sticky trap designs were designed to be supported by the previously described pipe cleaner set up to minimized weight and stress on the leaf. Healthy, susceptible Jagger plants were chosen so that high aphid populations could be observed in the cages to determine if aphid crowding, condensation, or mildew would be a factor in cage design. With the exception of the sticky trap, all cages were designed to enclose an entire leaf to allow the maximum amount of space for aphids to feed and reproduce.

The first cage design consisted of the previous method of clipping all but one 15.2 cm leaf (the second or third leaf to emerge) from the plant. Five large *D. noxia* nymphs were then placed on a square of paper at the base of the plant and the entire pot was caged in a 75 micron nylon screen mesh cage (Tuppro Brand, 18.9 liter, Redmond, WA). New leaf growth was trimmed from these plants during the experiment to encourage aphids to colonize the single leaf.

In the second design, one 15.2 cm leaf (the second or third leaf) was covered with a #117 glassine pollination envelope (19.3 cm by 5.1 cm. Lawson Bags, Northfield, Il) that had been punctured with pins to provide aeration. The envelope was sealed at the base of the leaf with paper clips and five late instar *D. noxia* nymphs were placed on a square of paper that was placed inside the sleeve with tweezers. The open sleeve end was then sealed with overlapping paper clips, and a pipe cleaner was twisted around a plastic stake to provide a support "T" for the leaf and the envelope cage.

The third cage tested consisted of a 2.5 x 1.3 cm sticky trap placed over a 2.5 cm leaf. Because the oval trap opening was wider than the leaf, cloth netting was placed on the bottom of the trap to prevent aphids from escaping. Five late instar *D. noxia* nymphs were then placed directly on the leave and the top of the trap was sealed with cloth netting. Each leaf was supported with a pipe cleaner "T" support. Because aphids were confined to a smaller space, symptoms developed more quickly and aphids were allowed to feed for only 11 days.

The fourth cage evaluated was a 3 cm diameter plastic drinking straw punctured with pins to provide aeration. Cages were placed over an individual leaf and paper towel wadding was placed at the base of the leaf/straw to prevent aphid escape. Five large *D. noxia* nymphs were placed on a square of paper that was then rolled up and placed into the top open end of the straw. The straw was then sealed with paper towel wadding and supported with a pipe cleaner "T" support. Data were analyzed using the SAS Proc GLM procedure (SAS Institute 1985). Differences between treatment means were separated using LSD tests and tested for significance at $\alpha = 0.05$.

EST Chromosome 7DS Bin Mapping using Wheat Deletion Lines

EST sequences were identified for six genes putatively related to *D. noxia* biotype 1

resistance by analyzing microarray gene expression levels in resistant and susceptible plants (Table 2.1). Before using any in VIGS, the location of each on the short arm of chromosome 7D was confirmed using Chinese Spring deletion line plants missing physical bin sections of chromosome 7D. Seed of each deletion line were obtained from John Raupp, K-State Department of Plant Pathology Wheat Genetic and Genomic Resource Center. Deletion lines tested were del7DS3*del7DL8 (missing bin 3 on the short arm, bin 8 on the long arm of 7D), del 7DS2 (missing bin 2 on 7DS), del 7DS4 (missing bin 4 on 7DS), del7DS5 (missing bin 5 on 7DS), Chinese Spring (complete), DT7DS (ditelosomic), and N7D-T7B (nullisomic, tetrasomic AABBBB). Seed were grown in the greenhouse under previous conditions. DNA was extracted (CTAB protocol, Doyle and Doyle 1987, Cullings 1992) from each deletion line and used as a PCR template to amplify primers designed from different EST sequences. Primers were designed from the six candidate ESTs using the Primer3 website.

Table 1.1 Mean expression (fold change) of wheat genes in Affymetrix gene chip hybridizations with cDNAs from uninfested plants of *D. noxia* resistant (*Dnx0*) plants and *D. noxia* susceptible (*S0)* plants and *D. noxia* resistant plants infested with B1 (*Dnx1*) or B2 (*Dnx2)* and *D. noxia* susceptible plants infested with B1 (*S1*) (Smith et al. 2010).

Affymetrix Wheat Gene Chip EST ID	**Gene Description**	***Dnx1/ Dnx0***	***Dnx0 /S0***	***Dnx1 /S1***	***Dnx1 /Dn2***
Ta.7883.1.S1_x_at	Putative disease resistance (DR) (dirigent-related) protein	0.45	30.44	15.72	1.16
TaAffx.28897.1.S1_at	Putative NBS-LRR type DR protein	1.09	15.5	20.19	1.37
TaAffx.7388.1.S1_at	Putative DR protein	2.58	15.15	3.21	7.18
Ta.7963.2.S1_x_at	DR protein-like protein	0.69	10.45	7.31	1.71
Ta.12396.1.S1_at	NBS, putative 32.6 kDa jasmonate induced protein	2.37	4.253	1.36	2.99
TaAffx.108556.1.S1_x_a	Pathogenesis-related protein	22.81	.07	1.33	1.19

Polymerase chain reaction reactions were prepared as aliquots of 25 µl containing 6.5 µl nuclease free water, 12.5 µl of PCR mix (Qiagen, Valencia, CA), 2 µl left primer, 2 µl right primer, and 2 µl template DNA. Control samples were prepared as above, but modified to contain 2 additional µl of H_2O and no DNA. Samples were placed in PTC-100 Programmable Thermal Controller for Program "B" (Step 1 94°C for 3 min., 94°C for 1 min., step 3 65°C for 1 min. and -2°C per cycle after that, 72°C for 1 min., repeat step 27 times, 94°C for 1 min., 52°C for 1 min., 72°C for 1 min., repeat step 6 30 times, 72°C for 5 min., and 4°C for 24 hrs.). PCR

products were visualized on 2% agarose gel with Invitrogen (Invitrogen, Carslbad, CA) 100 BP ladder as a size standard.

Primers were designed from EST sequences identified in microarray experiments that exhibited higher expression in uninfested *Dnx* resistant plants than in uninfested susceptible *Dn0* plants. Sequences that successfully amplified CS DNA template were then tested with CS 7DS deletion line primers to determine if they were located on 7DS. Primers that amplified DNA from plants missing 7DS were removed from consideration.

VIGS Candidate Gene Selection

All of the original six candidate genes for VIGS amplified DNA template that was missing the chromosome arm thought to be the location of the *Dnx* gene. Further data analysis was used to select candidates. Putative ESTs to be evaluated were selected based on results of Smith et al. (2009), who evaluated general and specific gene responses in wheat plants containing the *Dnx* gene for resistance to *D. noxia* biotype 1. Genes were selected based on putative function and increased expression in resistant plants compared to susceptible plants. The four ESTs chosen (Table 1.2) included the isoflavone reductase homolog BE560566 (consensus wheat TaAffx.129494.1.S1_at) which functions in regulation of nitrogen utilization and oxidoreductase activity and is located on wheat chromosome 7D Hossain et al. (2004); TaAffx.104814.1.S1_at and TaAffx.28897.1.S1_, that have putative functions similar to NBS-LRR type disease resistance proteins which were elevated in expression in *Dnx* plants compared to susceptible plants lacking *Dnx*, and the allene oxide cyclase gene (AOC) TA.7703.1.S1_A_AT, from the JA pathway, also expressed in response to *D. noxia* feeding (Smith et al. 2009) (Table 1.2). TaAffx.104814.1. S1_at and TaAffx.28897.1.S1_ were referred to as NBSLRR1 and NBSLRR2, respectively, in all experiments..)

Barley Stripe Mosaic Virus Preparation and Construction

BSMV viral RNA and BSMV:PDS viral RNA was constructed and inoculated into plants. Five *Dnx* plants were inoculated with BSMV on the third leaf. The silenced leaf was enclosed in a plastic straw cage and infested with seven aphids. Phytoene desaturase (PDS) silenced plants were not infested, but the photobleaching symptom was used as a visual marker to indicate that silencing occurred. BSMV plasmids containing clones of alpha (α), beta (β), gamma (δ), and gamma (δ) PDS genomes were obtained from Dr. Li Huang, Department of Plant Sciences and Plant Pathology, Montana State University. Genomes were transferred into chemically competent cells using Invitrogen One Shot TOP10 Chemically Competent *E. coli* cells similar to DH10B strain, and competent cells were grown on LB plates (LB agar media + 100 mg/L carbinicillin) at 37°C overnight. Single isolated colonies were then picked and grown at 37° C overnight in liquid culture (LB + 100 mg/L carbinicillin). Plasmids were then purified using QIAGEN QIAprep Centrifuge Miniprep Kit following product protocol without the addition of RNase to buffer P1. Plasmid quality was verified on 1% agarose gel and concentration was checked with a Thermo Scientific NanoDrop 1000 spectrophotometer before proceeding.

Plasmids with acceptable concentration were then linearized with respective enzymes for α, β, δ, and δ PDS. After digestion, plasmids were used to create RNA from the Ambion Maxiscript T7 transcription kit according to protocol for creation of capped transcripts. Quality was confirmed on a 1% agarose gel and transcripts were stored at -80 °C until inoculation.

Prior to inoculation, FES buffer was mixed, autoclaved and used as the buffer for all inoculations. GP buffer was prepared by combining 18.77 g glycine and 26.13 g K_2HPO_4 with enough ddH_2O to equal 500 ml. FES buffer was prepared by adding 100 mL of GP buffer to 5

g of sodium pyrophosphate decahydrate, 5 g of bentonite, and 5 g of celite and brought to 500 mL with ddH_2O. Buffer was then divided into 50 mL aliquots and autoclaved.

Sixty µl of transcript (20 µl each of α, β, δ, or δ PDS) was combined with 440 µl of FES buffer. For the first plant, 25 µl of BSMV:PDS was placed on a gloved index finger and dabbed with the thumb three times. The third leaf of the *Dnx* plant was then drawn between the index finger and thumb three times. A labeled stake was placed in the pot to mark inoculated plants. The process was repeated with 22 µl of BSMV:PDS for each *Dnx* plant. The inoculation procedure was then repeated for each *Dnx* BSMV inoculated plant. A total of seven plants (replications) per treatment were inoculated. Of these, five plants of similar size were infested with seven large *D. noxia* nymphs that were allowed to feed for 10 days. At 20- and 25 days post inoculation, chlorophyll A + B content readings were taken from the fourth and fifth leaves of the no virus *Dnx* plants and from the silenced (fifth) leaf and symptomatic (fourth) leaves of the BSMV inoculated *Dnx* plants, using a SPAD 502-chlorophyll meter (Minolta Corp.). Five separate SPAD meter readings were taken at various points along the leaf and averaged to obtain the chlorophyll amount for one leaf. Data were analyzed using the SAS Proc GLM procedure (SAS Institute 1985). Differences between treatment means were separated using LSD test and tested for significance at $\alpha = 0.05$.

Table 1.2 Mean expression (fold change) of wheat NBSLRR1, NBSLRR2, allene oxide cyclase, and isoflavone reductase genes in Affymetrix gene chip hybridizations with cDNAs from uninfested plants of *D. noxia* resistant (*Dnx*) plants (n=3) and *D. noxia* susceptible (*Dn0)* plants (n=2) (Smith et al. 2010)

Gene	Affymetrix Wheat Gene Chip EST ID	Mean up-regulation (fold change)	
		Dnx plants	*Dn0* plants
Isoflavone reductase	TaAffx.129494.1.S1_at	7	1.5
NBSLRR1	TaAffx.104814.1.S1_at	11.3	6.6
NBSLRR2	TaAffx.28897.1.S1_at	8.5	3.9
Allene oxide cyclase	TA.7703.1.S1_A_AT	9.2	10.0

Silencing of NBSLRR1 and NBSLRR

To prepare the α, β, and δ BSMV genomes (for BSMV with no gene inserted), the protocol previously stated for Barley Stripe Mosaic Virus preparation and construction was followed (see page 12). BSMV plasmids containing clones of α, β, and δ genomes were obtained from Dr. Li Huang at KSU/Montana State. Viral RNA was prepared and inoculated into plants using the previously described protocol. PCR primers for NBSLRR1 and NBSLRR2 EST sequences in Table 1.1 were designed using Beacon software. Sequences were amplified using *Dnx* genomic DNA as template under the following PCR reaction protocol: 12.5 µl PCR mix, 2.0 µl Primer 1, 2.0 µl Primer 2, 2.0 µl *Dnx* DNA template, and 6.5 µl H_20. Control samples were prepared as above, but modified to contain 2 additional µls of H_2O and no DNA. Samples were placed in PTC-100 Programmable Thermal Controller for Program "B" (Step 1

94°C for 3 min., 94°C for 1 min., step 3 65°C for 1 min. and -2°C per cycle after that, 72°C for 1 min., repeat step 27 times, 94°C for 1 min., 52°C for 1 min., 72°C for 1 min., repeat step 6 30 times, 72°C for 5 min., and 4°C for 24 hrs.). PCR products were visualized on 2% agarose gel with Invitrogen (Invitrogen, Carlsbad, CA) 100 BP ladder as a size standard. PCR products were purified according to protocol with Cycle Pure PCR Purification Kit (Omega Biotek, Frederick, CO).

The δ-PCR vector was used to clone the NBS1 and NBS2 fragments into the genome. Gamma-PCR is similar to δ except the PCR vector contains a recognition site for a PCR fragment. Gamma-PCR plasmid stock was provided by Xuming Liu, Department of Entomology, Kansas State University. Plasmids were grown overnight in LB media containing carbinicillin and then purified according protocol with Qiagen Miniprep kit. Concentration and quality were verified using a Thermo Scientific NanoDrop 1000 spectrophotometer, and a 1% agarose gel. Plasmids were linearized using *XcmI* and completion of digestion was checked on a 1% agarose gel. Linearized plasmid was then purified using a Cycle Pure Kit and eluted into 20 μl water. To check vector quality the linearized gamma-PCR vector was test ligated with T4 enzyme at 16° C overnight, cleaned by dialysis against water, and then transformed into bacteria using the Invitrogen One Shot TOP10 Chemically Competent kit. 25 ul were plated on agarose plates (LB agar media + 100 mg/L carbenicillin) and if less than 20 colonies were present after overnight growth, the vector was considered suitable for further cloning.

PCR fragments were generated by Beacon Designer Software from the suitable vector was then combined with purified PCR fragments and incubated at 16° C overnight. The δ - PCR/gene of interest, and α and β genomes were prepared as follows. Genomes were transformed into competent cells using a One Shot TOP10 kit according to the manufacturer's

protocol and plated overnight. Individual colonies were then picked with a pipette tip and swirled in a PCR tube containing 10 µl nuclease-free H_20; tip was then transferred to a 50 ml conical tube containing 15 ml LB media (LB media + 100 mg/L) and grown overnight at 37 ° C with shaking at 225 rpm. The water tube was then combined with 6.15 µl PCR mix, 1.0 µl P1, 1.0 µl P2, and 1.85 µl nf H_2O. Tubes were then placed in the thermocycler for the following cycle: Step 1 - 95° C for 3 min.; Step 2 - 95° C for 30 sec.; Step 3 - 53° C for 1 min.; Step 4 - 72° C for 1 min.; Step 5 - Repeat Steps 2-4 for 29 times; Step 6 - 72° C for 3 min.; Step 7 - 4° C to end. This procedure was performed for each plate containing either δ-PCR/NBSLRR1 or δ-PCR/NBSLRR2. Four clones were picked and amplified for each gene. PCR results were checked on 1% agarose gel and colonies that successfully amplified a ~175 bp fragment were considered successful clones. Successful clones were then linearized using 6 units of BssHII at 50° C for 90 min. and 80° C for 20 min. and digestion was checked on a 1% gel. Successful digestions were treated with RNase inhibitor (1 µl per 20 µl digestion) and then used for RNA transcription. Transcription was carried out using Ambion T7 Maxiscript Kit with the addition of Cap Analog mix. Reaction components were: 2.5 µg DNA template, 3.55 µl H_2O, 1.25 each of 2 mmol ATP, UTP, and CTP; 1.25 µl of 1 mmol GTP, 1.25 µl 10 mmol cap analog mix, 2.5 µl T7 buffer, and 2.5 µl T7 enzyme. Reactions were carried out at room temperature and the T7 buffer was allowed to warm to room temperature to avoid DNA precipitation. Reactions were incubated at 37° C for 4 hours and completion was checked on a 1% TBE gel. Successful transcriptions were stored at -80° C prior to inoculation. Sterile FES buffer was used as a buffer for all inoculations. GP buffer was prepared by combining 18.77 g glycine and 26.13 g K_2HPO_4 with enough ddH_2O to equal 500 ml. 100 mL of GP buffer was then added to 5 g of

sodium pyrophosphate decahydrate, 5 g of bentonite, and 5 g of celite and brought to 500 mL with ddH_2O. Buffer was then divided into 50 mL aliquots and autoclaved.

Plant inoculations were carried out as previously described with the following modifications. *Dnx* plant treatments were barley stripe mosaic virus infected controls, uninfected controls, and plants inoculated with virus containing the silenced NBSLRR1 or NBSLRR2 genes. The susceptible *Dn0* plant treatments included virus-infected and uninfected controls.

Aphids were enclosed in plastic straw cages as previously described and allowed to feed. At 21 days post-virus inoculation (9 days post-infestation) aphids were removed from plants and stored in 70% ethanol in a microcentrifuge tube. A piece of leaf tissue approximately 2.5 cm from the distal end of the fourth leaf from each plant was also collected, flash-frozen in liquid nitrogen, and stored at -80° C for RNA extraction. Plants were then left in the growth chamber and additional tissue was collected at 30 days post-inoculation to evaluate if virus and gene silencing are effective for longer than 21 days. After aphids had been counted, excess ethanol was removed from each microcentrifuge tube until approximately 500 µl 70% ethanol remained in the tube. Open tubes were then placed in a 65° C water bath for 6 to 8 hours to allow excess ethanol to evaporate. To ensure complete dryness, the tubes were then placed in a 37° C oven for 6 to 8 hours. Each tube with aphids was weighed, the weight recorded and aphids were then brushed out of the tube and the weight of tube alone was recorded. The difference was recorded as the weight of aphids for each treatment. Weight data were analyzed using the SAS Proc GLM procedure (SAS Institute 1985). Differences between treatment means were separated using LSD tests and tested for significance at $\alpha = 0.05$.

RNA was extracted from frozen leaves using the Trizol method per manufacturer's protocol (Ambion, Foster City, CA). Quality was assessed using a 1% TBE gel and Thermo Scientific NanoDrop 1000 Spectrophotometer (Thermo Scientific, Wilmington, DE) readings. In addition RNA was then purified with the Qiagen RNeasy Plant Mini Kit (Qiagen, Valencia, CA) per manufacturer's protocol.

Verification of Silencing with PCR

TRI reagent (Molecular Research Center, Inc, Cincinnati, OH) was used with the manufacturer's protocol to harvest RNA from leaf tissue of NBSLRR1 silenced leaves, NBSLRR2 silenced leaves, BSMV silenced leaves, and uninfected *Dnx* and *Dn0* leaves. A NanoDrop™ 1000 spectrophotometer (Thermo Scientific, Wilmington, DE) was used to determine the quality and concentration of RNA. An additional cleaning step using the RNeasy Plant Mini Kit (Qiagen, Valencia, CA) was done to improve the 230/260 ratios. RNA was then purified with TURBO™ DNA-free (Ambion, Austin, TX) and 1 µg of RNA was reverse transcribed into cDNA using the iScript™ Select cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA). Gene silencing was confirmed using cDNA as template with primers for either NBSLRR 1, NBSLRR 2, Gamma sequence from BSMV, or the housekeeping gene (actin). The following PCR reaction protocol was used: 6.25 µl PCR mix, 1.0 µl Primer 1, 1.0 µl Primer 2, 1.0 µl *Dnx* DNA template, and 3.25 µl H20 and samples were placed in PTC-100 Programmable Thermal Controller for Program "B" (Step 1 94°C for 3 min., 94°C for 1 min., step 3 65°C for 1 min. and -2°C per cycle after that, 72°C for 1 min., repeat step 27 times, 94°C for 1 min., 52°C for 1 min., 72°C for 1 min., repeat step 6 30 times, 72°C for 5 min., and 4°C for 24 hrs.). PCR products were visualized on 1% agarose gel with Invitrogen (Invitrogen,

Carlsbad, CA) 100 BP ladder as a size standard. Concentrations were verified using NanoDrop™ 1000 spectrophotometer (Thermo Scientific, Wilmington, DE).

Results

VIGS Feasibility Study

At 10 days post-*D. noxia* infestation there were no significant reductions in aphid numbers among *Dnx:*$BSMV_{Defensin}$ plants, *Dnx*:BSMV plants, or non infected plants (Table 1.3). Silencing the defensin gene, which is thought to be active in Hessian fly resistance, had no effect on *D. noxia* resistance in *Dnx* plants. The presence of the virus vector had no affect on *D. noxia* resistance as well. There were also no significant differences in numbers of *D. noxia* on Jagger plants receiving any of the three virus treatments, or between K92:$BSMV_{Defensin}$ plants and non-infected K92 plants (K92:BSMVwere not tested.) Significant differences did occur between some treatments. For example, *Dnx* plants and *Dnx*:BSMV plants produced significantly fewer aphids than uninfected Jagger plants or Jagger:BSMV plants, indicating that *Dnx* plants maintain their resistance in the presence of the VIGS mechanism. There were no significant differences between any of the susceptible K92 treatments and resistant *Dnx* treatments. Jagger uninfected plants had significantly more aphids than K92:$BSMV_{Defensin}$ but all other susceptible treatments were statistically similar regardless of virus or control treatment.

At 17 days post-*D. noxia* infestation there were also no significant differences between the *Dnx* and *Dnx:*$BSMV_{Defensin}$ plants(Table 1.3), indicating that the virus did not affect *Dnx* resistance to *D. noxia*. Significant differences were observed between the *Dnx:*$BSMV_{Defensin}$, and *Dnx* plants when compared to all susceptible controls (Jagger, K92, and K92:$BSMV_{Defensin}$)

except for Jagger:$BSMV_{Defensin}$. Jagger:$BSMV_{Defensin}$ had significantly fewer aphids than any other susceptible control and was no different from either resistant control. Silencing the Defensin gene in Jagger resulted in a decreased number of aphids at 17 days (Table 1.3).

Table 1.3 Mean ± SE number of D. noxia biotype 1 aphids on resistant (Dnx) plants, and susceptible Jagger and Karl 92 plants containing no barley strip mosaic virus (BSMV) (uninfected controls), BSMV with the silenced defensin gene inserted, or BSMV infected controls, infested with five fourth-instar D. noxia at 10 and 17 days post infestation

Plant Genotype/Virus Treatment[1]	**n**	**Mean ± SE** [2] **number of aphids at 10 dpi**	**n**	**Mean ± SE** [3] **number of aphids at 17 dpi**
Jagger uninfected	5	47.6 ± 10.3 a	3	209.0 ± 18.7 a
Jagger:BSMV	2	36.0 ± 19.0 ab	n/a	n/a
K92 uninfected	11	26.9 ± 5.1 abc	4	199.5 ± 12.6 a
Jagger:$BSMV_{Defensin}$	4	24.5 ± 3.9 abc	3	33.0 ± 10.0 c
K92:$BSMV_{Defensin}$	3	18.3 ± 5.8 bc	2	117.5 ± 57.5 b
*Dnx:*BSMV	3	14.0 ± 2.3 bc	n/a	n/a
Dnx:$BSMV_{Defensin}$	5	10.6 ± 5.2 c	4	43.0 ± 7.5 c
Dnx uninfected	18	8.6 ± 1.9 c	6	41.5 ± 9.1 c

[1] Means followed by a different letter differ significantly at $\alpha = 0.05$, Duncan' test, based on ANOVA of transformed data

[2] Collected December 8th

[3] Collected December 15th

D. noxia Single Leaf Cage Type Study

The sticky trap and whole plant/single leaf cage designs permitted similar numbers of *D. noxia* to develop, despite the fact that aphids were collected from the sticky trap cages 7 days earlier than single leaves caged as a whole plant (Table 2.2). Although fewer aphids developed in either of these cages than in the paper sleeve and plastic straw cages, differences were non-significant except between the plastic straw and whole plant/single leaf cages. The low numbers of aphids present on plants in the whole plant/single leaf cages indicates that this cage is the least favorable method of caging *D. noxia* for population development experiments.

The plastic straw cages allowed the greatest *D. noxia* population accumulation, and based on these results, this cage was used to assess *D. noxia* population development on BSMV inoculated silenced (healthy) leaves of *Dnx* plants compared to healthy leaves from non-inoculated plants.

Table 1.4 Mean ± SE number of *D. noxia* biotype 1 on Jagger wheat plants at 18 days after infestation with five large *D. noxia* nymphs.

	Mean ± SE number of aphids [1]
Cage type	
Plastic Straw	175 ± 28 a [2]
Paper Sleeve	95 ± 43 ab
Sticky Trap [3]	47 ± 2 ab
Whole plant/single leaf	45 ± 21 b

[1] n = 2 ; F = 3.55, df = 7

[2] Means followed by a different letter differ significantly at α=.05, LSD test

[3] Collected after 11 days

BSMV Construction and Testing in Dnx Wheat Results

Dnx virus infected- and non-infected plants also showed no significant difference in numbers of *D. noxia* at 7 days post-infestation (Table 2.4). These results supported those in Tables 2.2 and 2.3 indicating that BSMV has no adverse effect on *Dnx* antibiosis, and that it may be used as a VIGS vector. Although uninfected susceptible control Jagger exhibited low numbers of aphids and a lack of chlorosis and leaf rolling, this could be due to the fact that aphids were only allowed to feed for 7 days, an inadequate interval of time for populations to build up. Such a shorter infestation time would also decrease the presence of aphid-induced chlorosis and leaf rolling. In the experiments shown in Tables 2.1 and 2.2, aphids were allowed to feed for 10 days and 17 days on potentially the entire plant. Aphids could have been introduced on the silenced leaf earlier in the experiment to better study population effects. In addition, the effect of trimming the symptomatic leaves could have added physical stress to the plant system.

There was no significant difference in the numbers of *D. noxia* produced on leaves of *Dnx* plants with silenced BSMV compared to uninfected *Dnx* leaves. The silenced plants exhibited an almost two-fold increase in numbers of *D. noxia* compared to their uninfected counterparts (Table 2.4). A similar trend was seen in Table 2.2 and 2.3 where *Dnx* plants inoculated with BSMV produced larger *D. noxia* populations compared to uninfected *Dnx* plants, but this data was also not significantly different. Viral inoculation seemed to decrease the silenced leaf's ability to suppress *D. noxia* population development. However, this trend was also present in susceptible Jagger BSMV infected plants (Table 2.4)

Table 1.5 Mean ± SE number of *D. noxia* biotype 1 on wheat plants containing the *Dnx* resistance gene and susceptible Jagger plants lacking a resistance gene after infection with barley stripe mosaic virus (BSMV) and in uninfected controls at 7 days post-aphid infestation

Plant Genotype/Virus Treatment	n	Mean ± SE number of aphids [1]
Dnx BSMV infected	5	22.2 ± 5.9 b [2]
Dnx uninfected control	5	13.3 ± 1.6 b
Jagger BSMV infected	5	41.3 ± 5.2 a
Jagger uninfected control	5	20.7 ± 4.4 b

[1] df = 15, F = 3.06

[2] Means followed by a different letter differ significantly at $\alpha = 0.05$, LSD test

At 20 days post infection (Table 2.4), the symptomatic leaves did have significantly lower chlorophyll content than the uninfected treatments. However, the silenced leaves were not significantly different from either the uninfected treatments or the symptomatic leaves. At 25 days post inoculation, uninfected control plants had significantly higher chlorophyll readings than BSMV symptomatic leaves, but the control readings were not different from those of the silenced leaves of the BSMV infected plants (Table 2.6). The silenced leaves were more similar to uninfected plants than to infected plants, demonstrating the effectiveness of the silencing process in the fifth leaf. Silencing is typically effective for up to 24 days.

Table 1.6 Mean ± SE chlorophyll A + B content of wheat plant leaves containing the *Dnx* resistance gene after infection with barley stripe mosaic virus (BSMV) at 20- and 25 days (d) post-virus inoculation

		Chlorophyll Content (Mean ± SE) [1]	
Plant Leaf /Virus Treatment	n	20 d post inoculation	25 d post inoculation
Uninfected fourth leaf control	5	28.4 ± .9 a [2]	28.4 ± .8 a
Uninfected fifth leaf control	5	28.5 ± 1.2 a	29.9 ± .9 a
BSMV infected silenced fifth leaf	5	24.7 ± 1.7 ab	26.2 ± 1.1 a
BSMV infected symptomatic fourth leaf	5	21.1 ± 3.4 b	19.0 2.6 b

[1] df = 18, F = 1.82, 20 d post inoculation; df = 16, F =3.98, 25 d post inoculation

[2] Means followed by a different letter differ significantly at $\alpha = 0.05$, LSD test

EST Chromosome 7DS Bin Mapping using Wheat Deletion Lines

All primers that amplified CS also amplified deletion line templates, including the CS nullisomic and ditelosomic lines. Based on these results it was not possible to identify candidate genes using PCR amplification, and a further analysis of microarray data was necessary.

BSMV Infection and PDS Silencing in Dnx Wheat

Plants inoculated with PDS were not infested with aphids as effective silencing would result in leaf photobleaching due to lack of PDS expression. The *Dnx* plants inoculated with BSMV showed no significant difference in aphid numbers compared to uninfected *Dnx* plants, although infected plants produced nearly twice as many aphids as uninfected plants. There was

no significant difference in mean numbers of aphids produced on either the infected or uninfected *Dnx* plants.

Table 1.7 Mean ± SE number of *D. noxia* biotype 1 on fourth leaves of wheat plants containing the *Dnx* resistance gene after infection with barley stripe mosaic virus (BSMV) at 10 days post-aphid infestation

Plant Genotype/Virus Treatment	n	Mean ± SE number of aphids [1]
Dnx BSMV infected	5	31.2 ± 3.3 a [2]
Dnx Uninfected Control	5	17.4 ± 3.3 a

[1] df = 4; F = 1.93,

[2] Means followed by a different letter differ significantly at $\alpha = 0.05$, Duncan test

Silencing of NBS-LRR1 and NBS-LRR2

SAS analysis revealed no significant differences among treatments based on aphid numbers (Table 2.7) except that *Dn0* uninfected treatments had significantly more aphids than all other treatments. The *Dnx* infected and uninfected controls were not significantly different from each other and the *Dn0* infected and uninfected controls were not significantly different from each other but the expected difference between *Dn0* infected and *Dnx* uninfected plants was not observed. In contrast to previous experiments, the infected controls for both resistant and susceptible genotypes did not exhibit twice as many aphids as their respective uninfected controls. NBS1 and NBS2 were not significantly different from each other or from any other treatment except for *Dn0* uninfected. It is likely that the 9 day feeding period was too short for

aphid populations to be impacted by the antibiotic effect of the *Dnx* plants and any silencing effect from NBS1 or NBS2.

Individual comparisons of mean weights of aphids produced on different plant genotype-virus treatments detected differences between some treatments (Figure 1-4). We expected to see all *Dnx* (resistant) treatments not significantly different from each other and all *Dn0* (susceptible) treatments not be significantly different from each other. In addition, we expected all *Dnx* treatments to be different from all *Dn0* treatments. The NBS1 and NBS2 silencing was conducted using *Dnx* plants as host plants. Therefore, there should be no difference in aphid weights between any *Dnx* treatment and NBS1 and NBS2 unless silencing of either gene occurred. The mean weights from susceptible controls *Dn0* infected and *Dn0* uninfected were not significantly differently from each other (Figure 1) and the resistant controls *Dnx* infected and *Dnx* uninfected were also not significant from each other (Figure 3). The expected significant differences in mean weight between resistant and susceptible controls were observed for the *Dnx* and *Dn0* uninfected controls ($P < 0.09$), for *Dn0* infected and *Dnx* uninfected plants ($P < 0.01$) and *Dn0* infected and *Dnx* infected ($P < 0.05$) . Mean weights of *D. noxia* from *Dn0* uninfected and *Dnx* infected plants were not signficantly different. In addition, the mean weight of aphids from *Dnx* $_{NBSLRR1}$ plants was greater than the mean weight of aphids on *Dnx* $_{NBSLRR2}$ plants (17.8 µg compared to 11.0 µg).

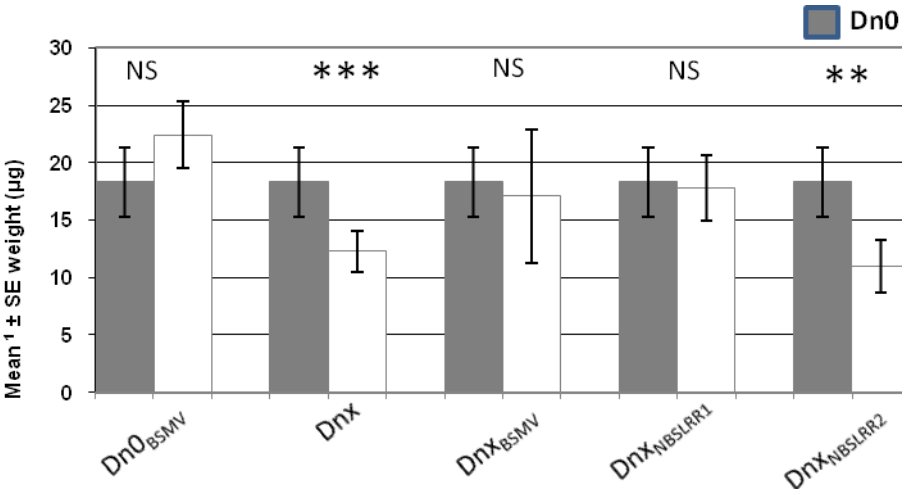
Table 1.8 Mean number of *D. noxia* biotype 1 aphids on *D. noxia* resistant wheat plants (*Dnx*) infected with barley stripe mosaic virus (BSMV), BSMV infected plants with the NBSLRR1 gene silenced, BSMV infected plants with the NBSLRR2 gene silenced, or uninfected virus control plants. Susceptible controls were BSMV infected- or uninfected *Dn0* plants. Plants were infested with 10 fourth instar stage *D. noxia* biotype 1 nymphs and aphids produced on plants were counted at 9 days post infestation

Plant Genotype/Virus Treatment	n	Mean ± SE number of aphids [1]
Dn0 uninfected control	11	59.5 ± 7.8 a
Dnx $_{\text{NBSLRR1}}$	13	40.9 ± 5.9 b
Dnx BSMV control	11	40.1 ± 5.6 b
Dnx uninfected control	12	39.9 ± 6.1 b
Dn0 BSMV control	15	38.7 ± 4.6 b
Dnx $_{\text{NBSLRR2}}$	12	38.6 ± 5.1 b

[1] df = 54, F = 1.02

[2] Means followed by a different letter differ significantly at $\alpha = 0.05$, LSD test

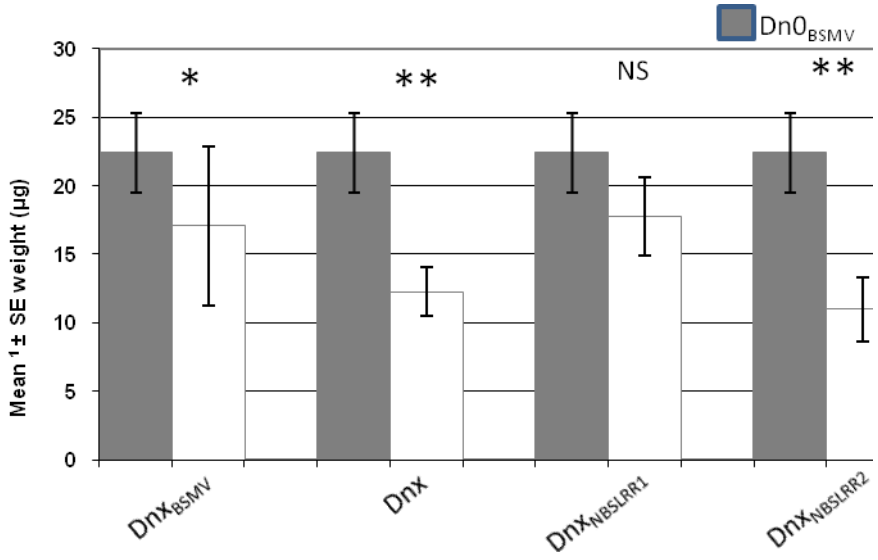
Figure 1 Differences in mean [1] ± SE weight (µg) of *D. noxia* biotype 1 reared on fourth leaves of uninfected *D. noxia* susceptible wheat plants (*Dn0*) compared to *Dn0* plants infected with BSMV, uninfected *Dnx* control plants, *Dnx* plants infected with BSMV, *Dnx* plants with the NBSLRR1 gene silenced or *Dnx* plants with the NBSLRR2 gene silenced at **9 days post infestation**



Significant - * $\alpha < 0.05$; ** $\alpha < 0.01$, *** $\alpha<0.09$, n. s. – non significant.

[1] Means and standard errors based are untransformed data, ANOVA based on transformed data

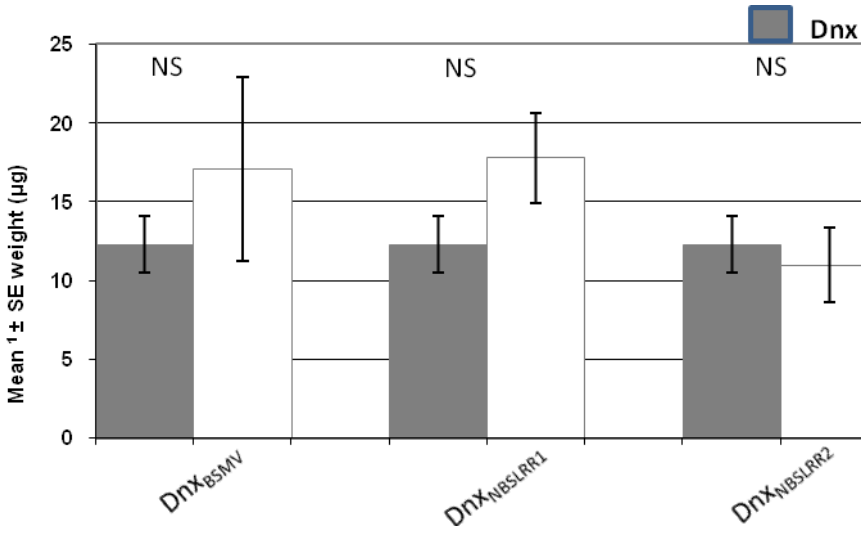
Figure 2 Differences in mean [1] ± SE weight (μg) of *D. noxia* biotype 1 reared on fourth leaves of *D. noxia* susceptible wheat plants (*Dn0*) infected with BSMV compared to silenced leaves from *Dnx* plants infected with BSMV, *Dnx* plants with the NBSLRR1 gene silenced and *Dnx* plants with the NBSLRR2 gene silenced at 9 days post infestation



Significant - * $\alpha < 0.05$; ** $\alpha < 0.01$, ***$\alpha < .09$, n. s. – non significant.

[1] Means and standard errors based are untransformed data, ANOVA based on transformed data

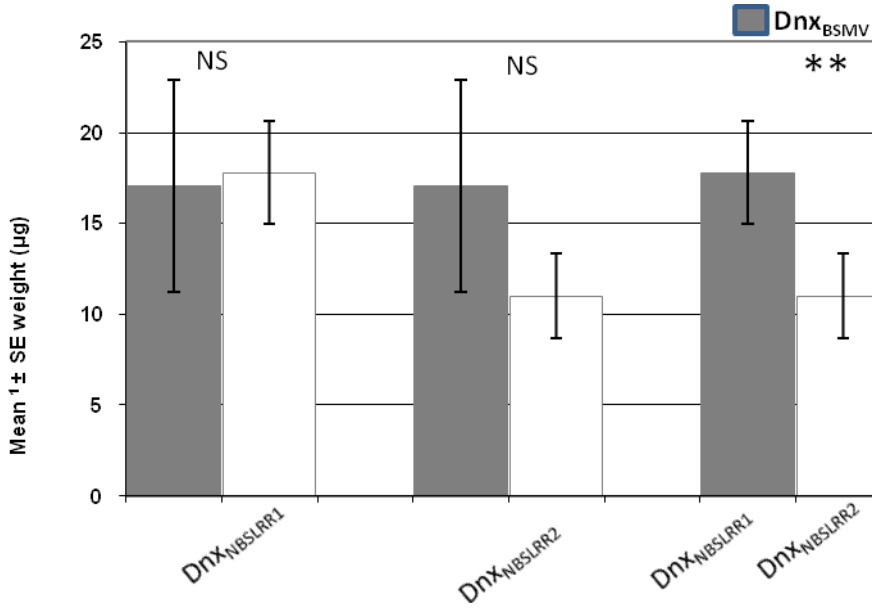
Figure 3 Differences in mean [1] ± SE weight (μg) of *D. noxia* biotype 1 reared on fourth leaves of uninfected *D. noxia* resistant wheat plants (*Dnx*) compared to silenced leaves from resistant *Dnx* infected with BSMV, *Dnx* plants with the NBSLRR1 gene silenced, and *Dnx* plants with the NBSLRR2 gene silenced at 9 days post infestation



Significant - * $\alpha < 0.05$; ** $\alpha < 0.01$, n. s. – non significant.

[1] Means and standard errors based are untransformed data, ANOVA based on transformed data

Figure 4 Differences in mean [1] ± SE weight (µg) of *D. noxia* biotype 1 reared on fourth (silenced) leaves of *D. noxia* resistant wheat plants (*Dnx*) infected with BSMV compared to *Dnx* plants with the NBSLRR1 gene silenced, *Dnx* plants with the NBSLRR2 gene silenced, and *Dnx* plants with NBSLRR 1 silenced compared to plants with NBSLRR 2 silenced at 9 days post infestation



Significant - * $\alpha < 0.05$; ** $\alpha < 0.01$, n. s. – non significant.

[1] Means and standard errors based are untransformed data, ANOVA based on transformed data

NBSLRR1 and NBSLRR2 silenced plants produced aphids with mean weights that were significantly different from each other and neither silencing treatment produced aphid weights that differed from either *Dnx* control plant treatment. NBSLRR1 silenced plants produced aphids with weights no different from either *Dn0* plant treatment while NBSLRR2 silenced plants produced aphids with weights that were significantly different from both *Dn0* plant treatments

Verification of Silencing with PCR

Gel analysis of PCR products detected some expression of BSMV in $Dn0_{BSMV:0}$ and $Dnx_{BSMV:0}$ plants at 21 days although in some replicates expression was very faint. PCR products of NBSLRR1 and NBSLRR2 were fainter in the silenced plants when compared to uninfected controls. At 30 days post-inoculation two of the three replicates showed less expression of NBSLRR1 and NBSLRR2 than the uninfected controls and it does appear that the gene expression remains reduced even after the 24 day periods of VIGS. Agarose gel analysis is not quantitative and does not reveal what the level of gene expression is and it is desirable to use Real Time PCR to quantify expression levels.

Figure 5 Expression of PCR reaction products in wheat leaf tissues sampled at 21 days post-BSMV inoculation and containing the silenced virus or silenced NBSLRR1 or NBSLRR2 genes.

Template	*Primer*	
$Dn0_{BSMV:0}$	BSMVγ	
Dn0	BSMVγ	
$Dnx_{BSMV:0}$	BSMVγ	
Dnx	BSMVγ	
$Dnx_{NBSLRR1}$	NBSLRR1	
Dnx	NBSLRR1	
$Dnx_{NBSLRR2}$	NBSLRR2	
Dnx	NBSLRR2	

Figure 6 Expression of PCR reaction products in wheat leaf tissues sampled at 30 days post-BSMV inoculation and containing silenced NBSLRR1 or NBSLRR2 genes.

Template	***Primer***	
Dnx$_{\text{NBSLRR1}}$	NBSLRR1	
Dnx	NBSLRR1	
Dnx$_{\text{NBSLRR2}}$	NBSLRR2	
Dnx	NBSLR2	

Discussion

VIGS Feasibility Study

At 10 days post-infestation, the number of aphids on *Dnx* treatment plants was not significantly from the number on *Dnx* controls and this trend was also seen in comparisons between the susceptible Jagger and K92 treatment versus and control plants (Table 1.3). These results indicate that *D. noxia* can survive on silenced plants as well as on healthy resistant- and susceptible control plants. All resistant *Dnx* plants had fewer aphids than susceptible plants, although the only significant difference observed between the three *Dnx* treatments and any of the susceptible treatments was the Jagger uninfected treatment. Thus, it is difficult to draw conclusions from data at 10 days post-infestation with a varying number of replicates.

At 17 days post-infestation, aphid populations were significantly lower on resistant treatments and populations still did not differ within resistant and susceptible treatments except for Jagger:$BSMV_{Defensin}$,plants, that had 33 ± 10 aphids per plant (Table 1.3). This population was significantly lower than that on all susceptible treatments but similar to populations on both resistant plant treatments. Silencing defensin in Jagger made the plants less suitable for aphid feeding. However, the experiment design was not standardized and the number of replicates per treatment varied from 2 to 19 and aphids were not confined to a particular leaf but were collected from various parts of the plant. Nevertheless, the fact that BSMV inoculation had no differential effect on aphid production on plants of different *Dnx* treatments suggests that VIGS can be utilized in wheat/aphid interactions using BSMV as a vector. The silencing effect is typically observed in only one leaf of a wheat plant and confining aphids to that leaf could have

also increased the precision of these experiments. These results led to the next series of experiments to test the effects of different ways of confining aphids to silenced leaves.

D. noxia Single Leaf Cage Type Study

The clipped leaf design appeared to exert stress on plant/*D. noxia* interactions because aphid populations produced in that cage were similar to those produced and caught on sticky traps (Table 2.3), despite the fact that the sticky traps covered a smaller area of the leaf and were collected 7 days earlier than the other designs. The significant reduction in *D. noxia* reproduction in the paper sleeve cages compared to plastic straw cages may be due to the fact that the sleeve cage was opaque, allowed little sunlight penetration, and had comparatively flat dimensions. The rounded diameter and clear color of the straw cage allowed more movement and light for the aphids compared to the paper sleeve cage. No mildew or condensation was observed in any of the cage designs. Khan et al. (2009) successfully used the plastic straw design to study *D. noxia* intrinsic rate of increase and this cage design is a viable method for confining aphids, especially to a particular leaf. Puterka et al. (2006) infested resistant susceptible barley lines with 20 adult aphids from several biotypes and found that susceptible varieties became 90% chlorotic at 14 days post-infestation with biotype 1 and that susceptible Morex plants supported a mean populations of 654 while resistant STARS 9301B had an average mean population of 971, although resistant plants had less chlorosis and leaf rolling symptoms. This experiment differs from our single leaf cages in that aphids were allowed to feed on an entire barley plant whole plant but it does indicate that susceptible plants could support a larger initial aphid infestation for at least 14 days.

EST Chromosome 7DS Bin Mapping using Wheat Deletion Lines

The primers for the candidate genes successfully amplified DNA template from all of the wheat deletion lines, including the line missing the short arm of chromosome 7DS (Table 1.1). Analysis of putative function and gene expression level were used to select NBSLRR1 and NBSLRR2 because of increased expression levels in *Dnx* plants compared to *Dn0* plants (Table 1.2) and because of previous work with the NBS-LRR gene *Mi-1* from the tomato plant that confers resistance to arthropods as well as nematodes (Milligan et al. 1998, Nombela et al. 2003). Scofield et al. (2005) used VIGS to study the effect of silencing the NBS-LRR wheat gene *Lr21 to* reveal its role in wheat pathogen resistance.

Barley Stripe Mosaic Virus Preparation and Construction

The defensin gene experiment indicated that a VIGS system using BSMV is viable in *Dnx* wheat plants. However, the results from these experiments with unequal numbers of replications indicated the need to find a method to confine aphids to only the silenced leaf. Therefore, an experiment was carried out to compare the *D. noxia* populations produced on *Dnx* and Jagger non-inoculated plants and plants inoculated with BSMV. To confine aphids, all leaves except for the silenced leaf were trimmed from the plant before infestation and trimming continued throughout the experiment to encourage aphids to feed on the silenced leaf. Results again indicated that there is no significant differences in aphid numbers produced on BSMV inoculated *Dnx* plants compared to non-inoculated plants (Table 2.6). In addition, *D. noxia* populations on both *Dnx* virus-infected and uninfected plants were significantly lower than susceptible Jagger virus-infected plants (Table 2.4) and no different from populations on non-infected Jagger plants. The *Dnx* infected plants produced had almost two times more aphids

than non-infected plants (Table 2.6) and this ratio was also present between the Jagger treatments in Table 2.4. The silencing mechanism did not appear to affect *D. noxia* survival, but perhaps another component of the experimental design affected the *D. noxia* population growth. For example, trimming symptomatic leaves from plants may have placed additional stress on the plant that could have affected aphid growth rate. In addition, an alternative method of confining *D. noxia* on a single leaf may have provided more consistent results.

VIGS Candidate Gene Selection

VIGS silencing should result in a leaf similar in appearance to leaves from healthy plants but visibly different from symptomatic leaves on the same plant, such as the BSMV symptoms of chlorosis and leaf mottling. As expected, the virus symptoms were expressed in the symptomatic leaves and they had significantly less chlorophyll than uninfected leaves (Table 2.5) and although we expected the leaf chlorophyll content of BSMV silenced leaves to be greater than BSMV symptomatic leaves, this was not the case at 20 days post-inoculation. However, at 25 days post-inoculation, the BSMV symptomatic leaves had significantly less chlorophyll than the uninfected fourth leaves, uninfected fifth leaves and also the BSMV silenced leaves. At 25 days,the plant has more time to accumulate chlorophyll and the silenced leaf would have a higher chlorophyll content than a leaf that has been exhibiting BSMV symptoms for 25 days.

Barley Stripe Mosaic Virus Infection and PDS Silencing in Dnx

Results of the previous experiments indicated that BSMV could be used to accurately assess the effect of silencing candidate genes in wheat for *D. noxia* resistance. In addition, plastic straw cages proved suitable to confine large *D. noxia* populations on non-inoculated

susceptible *Dn0* control plants which allowed the development of the highest aphid populations. The PDS silencing experiment was designed to test a larger number of inoculated *Dnx* plants and also to utilize the PDS gene to determine the length of time silencing remained in effect. In this experiment the PDS silenced plants were not infested with aphids because successful silencing would result in photobleaching that could deter aphid feeding (data not shown). However, the *Dnx* plants inoculated with BSMV were again not significantly different from their uninfected counterparts in aphid number. When caged with plastic straws as opposed to the leaf clip cages, inoculated plants again produced almost two times as many aphids as the uninfected control plants (Table 2.6), although the mean differences were not significantly different. These results suggest that although aphid populations are slightly greater on BSMV silenced plants than normal plants, silencing does not interfere with constitutive resistance of *Dnx* plants and the plastic straw cages provide a suitable environment for aphid population growth.

NBS-LRR 1 and 2 (2.7, 2.8-2.11)

Results of the virus vector, cage design, and candidate gene experiments were combined to evaluate NBSLRR1 and NBSLRR2 for their role in resistance to. Results of these gene silencing experiments (Table 2.7) differed from previous experiments (Tables 2.4, 2.6) in that non-inoculated resistant and susceptible control plants had higher *D. noxia* populations than inoculated controls although the values for *Dnx* infected and uninfected treatments were very similar. The only treatment that was significantly different was the *Dn0* uninfected treatment and it differed from all other treatments, including its infected counterpart (Table 2.7). NB1 and NB2 silenced plants were similar to both *Dnx* treatments and also the *Dn0* infected treatment and the expected differences between mean aphid populations on resistant and susceptible

controls were not observed in this experiment. It is difficult to draw conclusions about the effectiveness of silencing based on aphid population but the feeding period of nine days could have been too short to allow population differences to develop. Prolonged feeding period could be especially important in detecting population differences in *D. noxia* because they have been shown to have a longer development time and a lower fecundity and daily reproductive rate than other aphid species (Qureshi and Michaud 2005).

Mean weight per aphid was also measured to determine if the silencing treatments were affecting how much aphids were ingesting. BSMV VIGS does not appear to affect the natural resistance or susceptibility of wheat plants because the mean *D. noxia* weights on *Dn0* inoculated and non-inoculated control plants were not significantly different (Figure 1). The same was true for the corresponding *Dnx* treatments. In addition, mean *D. noxia* weights on *Dnx* plants were still significantly less than weights on susceptible plants in control experiments except for differences between *Dn0* uninfected and *Dnx* uninfected, which approached significance ($Pr < 0.09$) (Figures 1) and the *Dn0* uninfected treatment compared to the *Dnx* infected comparison which were not significantly different. *Dn0* uninfected plants had a lower mean weight than *Dn0* infected plant and *Dnx* uninfected plants also had a lower mean weight than their infected controls. Previous data suggests that aphid population is higher on silenced leaves (Tables 2.4, 2.6) but not significantly different from the respective uninfected controls. This could be due to the fact that aphids are feeding more on silenced leaves and aphids might gain more weight on resistant silenced leaves and the resulting weights could be similar to a susceptible plant. This would explain the higher weight per aphid on *Dnx* infected plants (17.07 μg) which is not significantly different from the weight on the *Dn0* uninfected plants (18.31 μg)

but still different from the weight of aphids on *Dn0* infected plants (22.41 μg) because those leaves were silenced and susceptible.

The fact that aphids had a higher mean weight on susceptible plants compared to resistant plants indicates that aphids feed more on susceptible plants than on resistant plants. Electronic penetration graph (EPG) analysis has previously documented the fact that RWA B1 spends more time in the sieve element phase on *Dn0* susceptible plants compared to *Dnx* resistant plants and thus ingests less phloem when feeding on resistant plants (Lazzari et al. 2009). Because mean weights of *D. noxia* produced on infected *Dn0* plants were no different from weights on non-infected *Dn0* plants and mean weights of *D. noxia* produced on *Dnx* infected plants were not significantly different from weights on non- infected *Dnx* plants (Figures 1 and 3) it does not appear that the VIGS mechanism deterred *D. noxia* feeding on plants within treatment controls.

Previous experiments (Tables 2.4, 2.6) revealed that resistant *Dnx* and susceptible *Dn0* plants supported higher *D. noxia* populations on BSMV-silenced leaves than their non-inoculated counterpart, but these differences were not significantly different within resistant and susceptible plant treatments. This trend was also present in the weights of *D. noxia*, where mean weights of *D. noxia* produced on *Dnx* inoculated plants were higher than weights of aphids produced on *Dnx* non-inoculated plants and weights of aphids produced on *Dn0* inoculated plants were higher than weights of aphids from *Dn0* non-inoculated plants (Figure 1).

Although silenced leaves seemed to support higher aphid populations and weights than non-silenced leaves, differences were not significant within resistant and susceptible plants and *Dn0* non-infected plants did not retain their expected significant differences from *Dnx* infected

and non-infected plants. The *Dn0* infected treatments did exhibit expected differences from *Dnx* infected and uninfected treatments, the mean weights were greater of aphids reared on susceptible *Dn0* infected plants (22.4 μg compared to 17.1 μg) on the *Dnx* infected plants (Figure 2). The lack of significance in aphid populations between all treatments and in weights between *Dn0* infected and *Dnx* infected plants could be due to the abbreviated 9 day aphid feeding period compared to other experiments, such as the 17 day experiment in Table 2.2. The shortened feeding period could have had a greater affect on aphid population than on mean aphid weight. Previous experiments have used feeding periods of 14 or 15 days (Lazzari et al. 2009, Khan et al. 2009) to detect population differences in resistant and susceptible wheat varieties to *D. noxia* biotypes 1 and 2. Experiments with biotype 1 aphids may allow an even longer feeding period than experiments with biotype 2 aphids because biotype 1 causes less severe symptoms than biotype 2 (Jyoti et al. 2006) and susceptible controls could be maintained along with resistant controls and treatment plants.

We expected the mean weights of *D. noxia* reared on NBSLRR1- and NBSLRR2-silenced *Dnx* plants to resemble weights of aphids reared on *Dnx* plants and to differ significantly from aphids reared on *Dn0* plants if silencing had no effect on *D. noxia* resistance. Although mean weights of aphids reared on NBSLRR1- and NBSLRR2-silenced plants were not different from weights of aphids on plants of other *Dnx* treatments (Figures 3 and 4), weights of aphids reared on NBSLRR2-silenced plants were significantly lower than those reared on either *Dn0* treatment (Figures 1 and 2), indicating that silencing NBSLR2 did not negate *Dnx* resistance. Weights of aphids reared on NBSLRR1-silenced plants were not significantly different from weights of aphids on either susceptible treatment but were greater than those of aphids reared on NBSLRR2-silenced plants and also *Dnx* infected- and uninfected

plants (Figures 1-4). Thus, silencing NBSLRR1appeared to have some effect on *Dnx* resistance even with a feeding period of only 9 days. From data presented here, it is possible to detect differences in *D. noxia* weight before differences in population numbers when resistance is due to antibiosis because aphids are spending less time feeding on resistant treatments and not gaining as much weight as their counterparts on susceptible treatments. Previously, aphid populations collected at 10 days (Table 2.2) and 7 days (Table 2.5) post-infestation on resistant and susceptible control plants were non-significant. It might be worthwhile to measure the weights of aphid populations even when population count is considered when resistance is antibiosis based.

In this experiment aphids were collected after only 9 days of feeding in the NBSLRR1 and NBSLRR2 silencing experiments because virus silencing was thought to cease after that point. Plants tissues were also collected when silencing was assumed to be over (30 days post-inoculation) to determine the presence of BSM virus in plants and if NBSLRR1 and NBSLRR2 remained silenced. If gene silencing is effective past 24 days post-inoculation then aphids could be allowed to feed on silenced plants for an extended period of time and it would be easier to detect differences in population numbers.

Verification of Silencing with RTPCR

Based on PCR and agarose gel analysis the VIGS mechanism did result in decreased levels of gene expression at both 21 and 30 days post-inoculation although in some cases suppression was not complete (Figures 5 and 6) and the results were not quantitative. Real Time PCR will be used to quantify the level of gene silencing.

Conclusions

The objectives of this project were to use bioinformatics and data mining to identify genes that could play a role in wheat resistance to *D. noxia* biotype 1, to evaluate BSMV as a VIGS vector in *Dnx* wheat and *D. noxia* interactions and to use VIGS to silence candidate genes in *Dnx* wheat and assess the silencing effect on *D. noxia* resistance. NBSLRR 1 and NBSLRR 2 seem to be viable candidates based on the expression level data from microarray analysis and because the NBSLRR genes are found in other resistance relationships. In the future it might be useful to examine genes from the JA pathway as this has also been indicated in *Dnx* resistance.

This experiment was unique in that we sought to utilize VIGS in a wheat-aphid interaction and to confine aphids only on the silenced leaf. In that respect we have demonstrated that BSMV is a viable vector for VIGS in wheat and that aphids can successfully feed on virus silenced leaves. We have also demonstrated that aphids can be successfully caged to a single leaf and that the perforated plastic straw design allows the largest populations to develop on susceptible plants. As silencing is only effective in one leaf, this design is more efficient than allowing aphids to feed on the entire plant.

Our data also indicates that aphids gain more weight when feeding on *Dnx* plants that have had the NBSLRR 1 gene silenced compared a resistant wheat plant with no silencing. We were not able to detect differences in aphid population numbers because of the brief feeding period, but our data does indicate that differences in mean weights are detectable before differences in population number. In the future it might be possible to use mean aphid weight as another method for measuring resistance. It was necessary to use a brief feeding period because the silencing mechanism is only effective for 24 days; however our PCR analysis shows that silenced plants exhibit decreased levels of the silenced gene even at 30 days post-

inoculation and it may be possible to extend the feeding period. Some of the replicates did not show a complete decrease in expression level and it would be desirable to use quantitative PCR to analyze expression levels.

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