THESIS

VIRUS-INDUCED GENE SILENCING AND MOLECULAR MARKER MAPPING FOR RUSSIAN WHEAT APHID RESITANCE IN WHEAT

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ABSTRACT

VIRUS-INDUCED GENE SILENCING AND MOLECULAR MARKER MAPPING FOR RUSSIAN WHEAT APHID RESITANCE IN WHEAT

Russian wheat aphids (RWA), *Diuraphis noxia* (Kurdjumov), are phloem-feeder insect pests that impart significant damage and yield losses upon infestation. They are a major economic threat to wheat (*Triticum aestivum* L.) and barley (*Hordeum vulgare* L.) in the western United States, having cost growers over $1 billion since their introduction in 1987. To date, eight RWA biotypes have been identified in the United States. RWA biotype 2 was identified in Colorado in 2003, is the largest threat to wheat growers and is virulent to almost all of the known resistance genes (*R*-genes). Continual emergence of new biotypes challenges the production of RWA resistant cultivars, since the race-specific, gene-for-gene resistance conferred by *R*-genes may be rendered ineffective with biotypic variation. Therefore, identification of durable resistance effective against multiple biotypes would be a significant advantage.

There are two sides of the resistance coin that can be explored for the development and deployment of wheat cultivars equipped with enhanced resistance. The first is a forward genetics approach involving the identification, characterization and implementation of new resistance sources. This includes novel *R*-gene discovery and

better understanding of resistance mechanisms, both of which can be enhanced by the integration of newly emerging genomic information and collaborative efforts to execute high-throughput evaluations of existing resistant germplasm. The other side of the resistance enhancement coin involves reverse genetics, investigating factors that contribute to cultivar susceptibility. Inhibition of essential products for pest/pathogen growth and development (*e.g.* appropriate nutrients or ease of access to phloem, in the case of aphids) via knockdown of genes involved in host plant susceptibility could produce resistance in previously susceptible genotypes. Investigations elucidating susceptibility factors are increasing in number and have the potential to be exploited for breeding resistance to diseases and pests. The overall goal of this study was to identify potentially novel sources of RWA resistance that could be utilized in wheat breeding programs for the production and deployment of cultivars with enhanced RWA resistance. To address this goal, two different aspects of host plant resistance were analyzed: investigation and characterization of a new *R*-gene for conference of *R*-gene-mediated resistance and expression reduction of a gene involved in the susceptible or compatible RWA-wheat interaction with the goal of inducing resistance in a previously susceptible genotype.

The first approach examined in this research was a reverse genetics investigation into susceptible RWA-wheat interactions. The goal was to test whether silencing a candidate gene predicted to be involved in compatible interactions between RWA and wheat would confer resistance to a susceptible wheat genotype. Several genes were identified as differentially expressed between the susceptible line, 'Gamtoos-S' (GS), and the near-

isogenic resistant line, 'Gamtoos-R' (GR; carrying *Dn7*), in a previous transcript profiling study (Botha et al., 2010). The goals of the current research were to identify a candidate gene up-regulated in compatible RWA-wheat interactions (GS compared to GR) and determine whether the gene is involved in these compatible interactions by assessing the effects of gene silencing on host symptom development and aphid reproduction. Barley stripe mosaic virus (BSMV)-mediated virus-induced gene silencing (VIGS) was employed to test whether *(1,3;1,4)*-β-*glucanase* is involved in the susceptible reaction of GS during RWA biotype 2 infestation. *(1,3;1,4)*-β-*glucanase* transcript abundance in the silenced treatment was reduced to levels similar to GR (P=0.600). Aphids on the silenced treatment reproduced less per day (P<0.0001) and had longer pre-nymphipositional periods than those on GS (P=0.003). Compared to GS, the silenced treatment exhibited less chlorosis (P<0.0001), greater dry weight (P=0.044) and had lower aphid to dry weight ratios (P=0.039). However, aphid-induced leaf rolling appeared to be unaffected by reduction of *(1,3;1,4)*-β-*glucanase* expression (P=1.000), suggesting separate mechanisms for leaf rolling and chlorosis. Aphid reproduction and host symptom development exhibitted linear relationships with *(1,3;1,4)*-β-*glucanase* transcript levels. VIGS construct sequence analyses indicated the possibility of non-target silencing of *(1,3)*-β-*glucanase*, but how transcript abundance of *(1,3)*-β-*glucanase* was altered by the VIGS construct designed for this study is yet to be determined. *(1,3;1,4)*-β-*glucanase* may be a susceptibility factor that could be exploited as a potential avenue of aphid resistance since suppression in the susceptible GS background to GR levels was related to a more RWA-resistant phenotype. Subsequent investigation into the possible co-silencing of *(1,3)*-β-*glucanase* and a more detailed examination of the role of

(1,3;1,4)-β-*glucanase* in RWA susceptibility will provide insight into how *(1,3;1,4)*-β-*glucanase* suppression might be used in the implementation of novel RWA resistance in wheat breeding programs.

A forward genetic approach involved examination of potentially novel resistance found in an Iranian wheat landrace accession that is resistant to RWA biotype 1 and the most virulent RWA biotype to date, RWA biotype 2. The objectives of this study were to determine the inheritance of resistance, identify closely linked markers, and map the chromosomal location of resistance found in Iranian landrace accession PI 626580. Bulked segregant analysis with a mapping population of 154-F_2 individuals, developed from a single plant selection made from PI 626580 and 'Yuma' (a susceptible wheat cultivar), was employed. Based on chi-square goodness-of-fit tests, RWA resistance in PI 626580 appears to be conferred by a single dominant gene, provisionally designated as *Dn10*. Linkage mapping analysis identified three SSR markers, *Xbarc214*, *Xgwm473* and *Xgwm437*, proximally linked to *Dn10* near the centromere on the short arm of chromosome 7D, at distances of 12.9 cM, 16.0 cM, and 19.2 cM, respectively. The marker order, with respect to resistance in PI 626580, was verified by a quantitative single factor analysis of variance which revealed significant marker associations with chlorosis and leaf rolling (P<0.0001 for all three markers). *Dn10*, a new resistance gene found in PI 626580 could be used alone or by pyramiding with other *Dn*-genes to develop cultivars with improved RWA resistance.

This research was aimed at investigating potentially novel sources of RWA resistance that could be utilized in wheat breeding programs. As a result, a RWA *R*-gene

provisionally designated as *Dn10* was identified and *(1,3;1,4)*-β-*glucanase* has been implicated as a susceptibility factor that could potentially be manipulated to confer RWA resistance to a susceptible cultivar. Further research will be needed to elucidate if and how the findings of these studies could be effectively implemented in the development and deployment of high yielding cultivars with enhanced RWA resistance. An integrated approach of employing forward and reverse genetics in the investigation of RWA resistance may ultimately be an effective strategy for combating the continuous cycle of biotype emergence and biotype-specific resistance.

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CHAPTER ONE

LITERATURE REVIEW

THE ORIGIN AND IMPORTANCE OF *TRITICUM AESTIVUM* L.

Wild relatives of bread wheat (*Triticum aestivum* L.), such as wild emmer (*T. dicoccoides*), have been utilized for tens of thousands of years, beginning before the advent of civilization (Fuller, 2007). With origins in the Near Eastern Fertile Crescent, post-domesticated wheat has become one of the world's most important food crops constituting almost 40% of total global grain production (IGC, 2010). According to the Food and Agriculture Organization of the United Nations, the United States is the fourth largest wheat producing country (FAOSTAT, 2010), producing over 54 million tons in 2009 (USDA-NASS, 2010). However, there is a growing food shortage, realized when production is compared to world population statistics. The global population is expanding rapidly and should reach 9 billion people by 2050 (USCB, 2008), raising serious questions about the adequacy of our food supply (Brown, 2009). Therefore, the production and distribution of higher yielding cultivars with improved tolerance to biotic and abiotic stresses is essential if we are to combat this eminent threat.

A better understanding of the wheat genome and the genetic interactions therein have the potential to aid in cultivar development by contributing to the discovery of novel genes and mechanisms of resistance. Bread wheat has three homoeologous genomes (2n=6x=42, AABBDD) (Sakamura 1918) merged together by the hybridization of *T. turgidum* L. (a tetraploid wheat formed ~0.5 Mya (Huang et al., 2002a), contributing the AABB genomes) and *Aegilops tauschii* Coss., a wild relative that contributed the DD genome (Kihara 1944; McFadden and Sears 1946), approximately 10,000 years ago (Figure 1.1) (Feldman et al., 1995; Feldman, 2001). *T. turgidum* L. resulted from hybridization of two diploid species; *T. urartu* (AA genome) and an unknown contributor of the BB genome, although the BB progenitor has been shown to most closely resemble *A. speltoides* (genome SS) (Blake et al., 1999; Feldman et al., 1995; Huang et al., 2002a). It is thought that the three diploid progenitors of hexaploid wheat diverged from their common ancestor about 4 Mya (Feldman, 2001). Chromosomal pairing within bread wheat is mainly limited to between homolog (within genome) pairing, not between homoeolog (among genome) pairing (Sears, 1976). The size (~17 Gb), complexity, and frequency of repetitive sequences (~80-90% of genome) present in this allohexaploid have made progress in understanding and utilizing the wheat genome slow (Choulet et al., 2010; Smith and Flavell, 1975; Wanjugi et al., 2009). This is illustrated when the wheat genome is compared to the genome sizes of two sequenced, model plant species: the wheat genome is about 40 times larger than the genome of rice (*Oryza sativa*) and 120 times larger than that of *Arabidopsis thaliana* (Lagudah et al., 2001).

THE RUSSIAN WHEAT APHID

Global crop production is continually threatened by losses due to pests and pathogens and wheat is no exception (Hesler et al., 2005; Colbach, 2010). Winter wheat is subject mainly to two-major viruses and a range of arthropod pests (Hesler et al., 2005). The Russian wheat aphid (RWA), *Diuraphis noxia* (Kurdjumov), is an important insect pest of wheat and barley (*Hordeum vulgare* L.) and has had a major economic impact worldwide, especially on winter wheat in the western part of the United States (Burd et al., 2006; Haley et al., 2004; Weiland et al., 2008). RWA was discovered in the Ukraine and Moldova in the early 1900s, but it wasn't until 1978, when it was identified in South Africa, that the potential for RWA-induced yield losses began to be understood (Halbert and Stoetzel, 1998). Only two years after the discovery in South Africa, RWA was found in Mexico by employees of the International Maize and Wheat Improvement Center (CIMMYT), where serious damage occurred (Halbert and Stoetzel, 1998). RWA first reached the United States in 1986 when found in Bailey County, Texas (Burd et al., 2006) and within a year, RWA was identified in six more states: Wyoming, Oklahoma, Nebraska, New Mexico, Kansas, and Colorado (Halbert and Stoetzel, 1998). It is now a threat in 16 western states (Figure 1.2) (Halbert and Stoetzel, 1998). Direct and indirect damages due to RWA in the western U.S. alone exceeded $800 million within the first seven years after its introduction (Morrison and Peairs, 1998).

A single biotype existed in the U.S. until a second, more virulent biotype (RWA biotype 2) was discovered in Colorado in 2003 and was recognized to be virulent to most RWA resistant sources at the time (Haley et al., 2004; Porter et al., 2005). Since the

discovery of RWA biotype 2, six more biotypes have been identified; three in Colorado, two in Texas and one in Wyoming (Randolph et al., 2008). Yet RWA biotype 2 remains the most virulent and the largest RWA threat to wheat and barley growers in the western U.S. (Randolph et al., 2008).

Methods used to control the RWA have included insecticide application (requiring complete coverage), bio-control agents (such as the aphid parasitoid wasp, *Diaeretiella rapae*), and the production and deployment of resistant cultivars (Baker et al., 2003; Burd et al., 2006; Hodgson and Karren, 2008; Morrison and Peairs, 1998). The environmental and economic cost of insecticide use and the possibility of the emergence of insecticide-resistant aphids are potential deterrents to chemical control of RWA (Burd et al., 2006). Bio-control has its own complications. RWA reproduction out-paces that of most of its bio-control species and since bio-control agent populations tend to expand more slowly, by the time they reach effective sizes RWA has already imparted significant damage to their hosts (Adisu and Freier, 2003; Tagu et al., 2008). Additionally, aphid feeding induces leaf rolling (discussed later), physically protecting RWA from contact insecticides and/or bio-control agents (Haile et al., 1999). Non-native introductions are at high risk for becoming invasive species that, due to difficulties in eradication, present a whole new set of challenges (Andreu and Vila, 2010). Therefore, production of resistant cultivars remains the most viable option for control. With the continuing emergence of new biotypes virulent to existing resistance genes, however, there is need for the discovery and implementation of more durable resistance.

RWA (Figure 1.3) are phloem feeders that can be distinguished from other cereal aphids by their short antennae, reduced cornicles, and green, spindle-shaped bodies (Hodgson and Karren 2008). Female RWA reproduce mainly asexually, giving birth parthenogenetically to live young (Figure 1.3) for a period of about 60-80 days (Hodgson and Karren, 2008). Yet some populations outside of North America give birth to oviparae which require male fertilization and overwintering of the eggs (Halbert and Stoetzel, 1998). To date, a male RWA has not been found in North America (Hodgson and Karren, 2008). RWA adults exist mainly as wingless morphs until migration or they become unsatisfied by the decline of the plant host, at which point winged morphs are produced (Halbert and Stoetzel, 1998; Tagu et al., 2008). These winged morphs serve as host selectors and their creation is spurred by external cues such as overcrowding and host deterioration, as well as abiotic cues like changes in temperature and photoperiod (Tagu et al., 2008).

RWA-HOST INTERACTIONS

RWA feeding

RWA feed on photoassimilates located in the phloem of their host plants via intercellular insertion of a stylet into the plants sieve elements (SE); although some cellular penetration may occur, causing plasma membrane damage of the mesophyll and parenchyma cells and cell wall disruption (Lapitan et al., 2007a; Miles, 1999; Moran et al., 2002; Will and Van Bel, 2006). The aphid stylet consists of both salivary and food canals, the former utilized for probing and plant response elicitation (Will et al., 2007). Aphids secrete two types of saliva through their salivary channel, gel-like saliva and

watery saliva, each with specific roles. The gel-like or sheath saliva creates a continuous sheath (immediately upon secretion) around the stylet (Tjallingii, 2006; Will and Van Bel, 2006). This secretion occurs just prior to and during stylet insertion and adheres the stylet to the SE, preventing loss of turgor pressure (required for sap flow within the phloem) by sealing the puncture site (Walling, 2008; Will and Van Bel, 2006). Additionally, this wound-site sealing acts to reduce Ca^{2+} influx in the apoplast, a plant defense response produced to coagulate proteins at the puncture site (reviewed by Will et al., 2007). The composition of sheath saliva consists of phospholipids, conjugated carbohydrates, free amino acids, and the activities of oxidative and 1,4-glucosidase enzymes with the ability to polymerize both plant- and insect-derived phenolics and proteins (Miles, 1999; Moran et al., 2002; Smith and Boyko, 2007). Sheath saliva may also sequester oxidized phenolic forms to suppress their accumulation, which is a response to wounding (Moran et al., 2002).

Watery or digestive saliva is secreted during cellular stylet penetration (probing) and at the beginning (and throughout the duration) of feeding; the latter for the purpose of combating SE responses to wounding (Tjallingii, 2006). The aphid secretes watery saliva that initiates sap intake and after successful penetration into the SE, helps prevent the coagulation of phloem proteins, similar to sheath saliva function; this secretion will continue until sap intake is terminated (Tjallingii, 2006; Will et al., 2007). The components of the watery saliva are a complex mixture of compounds that can trigger host plant defense responses including enzymes such as pectinase, cellulase, peroxidase, polyphenol oxidase, and lipase (Moran et al., 2002; Smith and Boyko, 2007). These

enzymes are predicted to serve many functions including stylet lubrication, detoxification of phenolics, redox condition maintenance and prevention of callose occlusion of SE that occurs through Ca^{2+}-binding (Moran et al., 2002; Will et al., 2007). The latter prohibits the attainment of threshold Ca^{2+} concentrations high enough to trigger this wound response.

Feeding at the base of the newest (youngest) leaves is preferred by RWA, since these areas are strong nutrient sinks, although any living tissue is suitable (Franzen et al., 2007). Concentration of ten essential amino acids in the phloem-sap substantially increases with an increase in RWA infestation time (Telang et al., 1999), providing the aphids with sufficient nutrients that were lacking prior to feeding (Giordanengo et al., 2010). Additionally, research has indicated that aphid feeding can result in the untimely alteration of the host sink-source relationship (Burd et al., 1996). Burd et al. (1996) explained that young leaves will convert from sink to source when about ½ of the photosynthetic capacity of the leaf has been reached, but that RWA feeding on photoassimilates, combined with chlorophyll loss, may alter the sink-status of the leaf to source. This was supported by other research where nitrogen sink-to-source switches, after extended aphid feeding, were observed in both infested and non-infested tissues (Giordanengo et al., 2010). Reports like these lend credence to the hypothesis that aphids are able to manipulate plant metabolism and host sink-source relationships for their own benefit (Burd et al., 1996; Giordanengo et al., 2010; Smith and Boyko, 2007).

Host plant response

Aphid salivary secretions are of great interest and importance when investigating host responses to aphid feeding since the eliciting agent is found within these secretions. In fact, aphid secretions have been likened to the type III secretion system of bacteria as the vehicle for response elicitation (Tagu et al., 2008). The RWA effector has been shown to be a protein and injection of RWA salivary protein fraction into the plant induces a cascade of defense responses leading to resistance-mediated responses in resistant plants or symptom development in susceptible plants (Botha et al., 2010; Lapitan et al., 2007a; Smith and Boyko, 2007). Despite knowledge that the RWA elicitor is in the protein fraction, identification of the specific effector has been unsuccessful thus far (Lapitan et al., 2007a). Once the identity of the effector is known, research involving effector-targets will aid in the understanding and utilization of enhanced RWA resistance. Until then we must make inferences from our knowledge of other plant-pest/pathogen interactions and evidence that emerging RWA research has been able to piece together. Host response to aphid feeding is unique, triggering defense signaling pathways that induce both pathogen-response genes and wound-response genes typically seen with chewing insects (Halbert and Stoetzel, 1998; Montesano et al., 2003; Smith and Boyko, 2007). In an attempt to protect themselves against the pathogen-like and wounding nature of aphid attack, plants recruit a complex system of crosstalk between pathways induced by or involving salicylic acid (SA), jasmonic acid (JA), ethylene (ET), reactive oxygen species (ROS) and in some cases nitric oxide (NO) (Figure 1.4) (Park et al., 2006; Rojo et al., 2003; Walling, 2008). That aphid feeding triggers both SA- and JA/ET-mediated pathways is interesting, since these pathways appear to be antagonistically induced (Botha et al., 2005; Dong, 1998;

Heil and Bostock, 2002). However, the extent to which each pathway is involved depends largely on the species of both the aphid and the host and whether the interaction is compatible or incompatible (Smith and Boyko, 2007; Walling, 2008).

SA, essential for the hypersensitive response (HR) and systemic acquired resistance (SAR) (both to be discussed later), induces signaling cascades that elicit biotrophic pathogen defense via expression of pathogenesis-related (PR) genes (Figure 1.4) (Rojo et al., 2003; Smith and Boyko, 2007). Host suitability in susceptible (compatible) interactions with some aphid species may be enhanced by SA accumulation and SA-mediated signaling (Thompson and Goggin, 2006). Boyko et al. (2006) reported that many PR genes, like chitinase and β-*1,3-glucanase*, associated with the SA-signaling cascade exhibited significant expression increases when aphids fed on susceptible sorghum (*Sorghum bicolor*) and celery (*Apium graveolens*) genotypes, but this increase was not observed with resistant aphid-infested wheat or sorghum. Yet, with SA-mediated induction of PR genes, HR and SAR, SA accumulation and pathways may play the reverse role of resistance enhancement in plant-aphid interactions involving other plant and/or aphid species (Rojo et al., 2003; Smith and Boyko, 2007). Mewis et al. (2005) observed that green peach aphids (*Myzus persicae* Sulzer) and cabbage aphids (*Brevicoryne brassicae*) did not perform as well on *A. thaliana* mutants of *npr1* and *NahG*; they observed significant reduction in total aphid numbers (fecundity). *NahG* suppresses SA accumulation by salicylate hydroxylase degradation of SA and *npr1* functions in a similar manner by reducing SA, yet also enhancing JA-mediated signaling (Mewis et al., 2005; Thompson and Goggin, 2006). This supports the hypothesis that that

SA-mediated signaling pathways may be involved in enhanced resistance to certain species of aphids (Thompson and Goggin, 2006; Zhu-Salzman et al., 2004).

JA and ET pathways help plants with pest/pathogen resistance by working cooperatively, synergistically, or sequentially, but almost always in consort (Rojo et al., 2003). JA pathway induction is negatively affected by inhibition of ET production and, conversely, post-injury production of ET is initiated by JA (and/or cell wall polysaccharide fragments) (Dong, 1998). Additionally, in *Arabidopsis*, PR genes such as chitinase (PR-3) and defensin (PDF1.2), as well as *ethylene response factor 1* (*ERF1*), require signaling from both JA and ET for induction and expression (Rojo et al., 2003). JA and ET are involved in necrotrophic and saprophytic pathogen defense (Dong, 1998; Rojo et al., 2003) in addition to wounding-response to chewing by the initiation of hydrogen peroxide accumulation (involved in ROS defense) and proteinase inhibitors that promote stunted growth and starvation of insects by inhibiting digestion (Smith and Boyko, 2007; Stotz et al., 1999). Genes involved in the octadecanoid pathway (Figure 1.4), leading to JA synthesis and JA-mediated defense responses have been implicated in resistance to insect herbivory and have been shown to be significantly induced by RWA and greenbug (*Schizaphis graminum*) feeding on aphid-resistant plants (Boyko et al., 2006; Park et al., 2006).

ET is also involved in production of allelochemicals, ROS, and HR (Smith and Boyko, 2007). The exact role of ET in aphid defense responses remains unclear, yet it has been associated with susceptibility to RWA and resistance to greenbug (Argandonã et al., 2001; Miller et al., 1994a; Thompson and Goggin, 2006), which emphasizes that the

involvement of the defense signaling pathways is not always a one-size-fits-all model for aphid-plant interactions (De Vos et al., 2007; Giordanengo et al., 2010; Smith and Boyko, 2007). The functionality of the specific pathways induced by aphid feeding varies depending on the plant and aphid species involved. Where SA-mediated responses may be up-regulated during aphid-infestation in resistant cultivars of some species, JA/ET-mediated pathways may play larger roles in the resistant reactions of other species (Goggin, 2007; Walling, 2008). Aphids may be manipulating the plant defense responses of pathway cross-talk by repressing the pathway(s) that provides the most effective resistance (for the specific species and/or aphid-host combination) by amplifying the antagonizing pathway (Smith and Boyko, 2007; Thompson and Goggin, 2006).

ROS function as direct elicitors of plant defense to pathogens and insects (Heil and Bostock, 2002; Smith and Boyko, 2007), including aphids (Boyko et al., 2006), through their induction upon the recognition of aphid salivary secretions within the cell (Thompson and Goggin, 2006). They also defend indirectly by having an adverse effect on the mid-gut tissues of arthropods (Heil and Bostock, 2002; Orozco-Cardenas and Ryan, 1999). The synthesis of hydrogen peroxide and other ROS is initiated by the degradation of linolenic acid, systemin, chitosan and oligogalacturonic acid and is also mediated through the octadecanoid pathway, the same pathway leading to JA synthesis (Figure 1.4) (Orozco-Cardenas and Ryan, 1999; Smith and Boyko, 2007). Although ROS have many proposed roles in pathogen defense like antimicrobial agents, the production of HR, SA, and ET, and enabling protein cross-linkage to inhibit spread of infection, it has been reported that ROS and RO intermediates often require the cooperation of NO to

fulfill some of these roles (Delledonne et al., 1998; Orozco-Cardenas and Ryan, 1999; Soosaar et al., 2005; Zhu-Salzman et al., 2004). NO has been shown to amplify ROS-induced hypersensitive cell death and activate expression of genes whose functions compliment those genes activated by ROS; this allows for NO- and ROS-specific defense involvement, but also provides incremental advancement of HR (Delledonne et al., 1998). Since NO doesn't appear to be involved in RWA feeding specifically (Botha et al., 2005; Smith and Boyko, 2007), it will not be covered in detail here. Despite the pest/pathogen defense function that necessitates ROS production, plants must balance ROS generation with ROS detoxification for plant tissue stabilization, otherwise they will suffer from their own oxidative damage (Smith and Boyko, 2007; Thompson and Goggin, 2006; Zhu-Salzman et al., 2004). Fortunately, this balance is usually met (Thompson and Goggin, 2006).

Abscisic acid (ABA) and gibberellic acid (GA) have both been linked to plant defense responses, but reports on their exact involvement is lacking. Precursors to ABA and GA, such as transketolase and aldehyde oxidase, are up-regulated under aphid-infested conditions (Boyko et al., 2006; Divol et al., 2005; Park et al., 2006). ABA's role in defense responses may be a function of its putative role in the octadecanoid pathway, affecting synthesis of JA (Smith and Boyko, 2007), in addition to its negative regulation of SA-dependent resistance (Rojo et al., 2003). GA-signaling has been shown to regulate the release of β-*1,3-glucanase*, a PR gene, from aleurone cells of germinated grain (Smith and Boyko, 2007). Other aphid-induced plant defense responses include allelochemical production and cell wall modifications. Allelochemicals, produced as a result of a

number of different signaling pathways (Figure 1.4), can be volatiles, which serve as insect repellents or deterrents, or non-volatiles, which negatively affect aphid feeding, growth, and reproduction (Smith and Boyko, 2007). Non-volatiles, like cytochrome P450 mono-oxygenase, have been shown to be highly up-regulated under aphid-infested conditions in wheat and sorghum (Boyko et al., 2006; Park et al., 2006). The exact roles of many of these compounds in defense is hard to determine due to the many functions they serve elsewhere in the plant (Smith and Boyko, 2007). Cell wall modification has been shown in pathogen-, nematode-, aphid- and herbivorous insect-related defense responses operating to strengthen cell wall barriers (Divol et al., 2005; Moran et al., 2002). In the case of aphids, the function of cell wall modification may expand to include facilitation of feeding through ease of stylet penetration and/or response to aphid-induced turgor pressure changes (Giordanengo et al., 2010).

Biotropic resistance mechanisms

Since the exact nature of RWA resistance is poorly understood, information from mechanisms involved in both pest and pathogen resistance are used as potential models for aphid resistance (Botha et al., 2006; Zhu-Salzman et al., 2004). There are three main forms of resistance that plants employ against biotrophic pests/pathogens: basal resistance, systemic acquired resistance (SAR), and resistance gene (*R*-gene)-mediated resistance, each capitalizing on similar signaling pathways and gene sets, playing a role in the evolutionary arms-race of virulence and avirulence (Anderson et al., 2010; Eulgem, 2005). Basal resistance, inducing basal defenses, is the earliest type of resistance response post-pathogen detection (Kiraly et al., 2007) and has been defined by Jones and

Dengl (2006) as resistance that comes about from "virulent pathogens on susceptible hosts". This type of resistance can suspend subsequent spread/colonization (Jones and Dangl, 2006), but does not prevent the growth and development of pathogens (Eulgem, 2005). Basal resistance is the first of a two-pronged system of plant innate immunity and provides general, non-specific resistance to pathogens (Jones and Dangl, 2006; Kiraly et al., 2007).

Basal resistance involves trans-membrane pattern recognition receptors (PRRs) that recognize and respond to slowly evolving, broadly conserved microbial or pathogenic features, referred to as microbial- or pathogen-associated molecular patterns (MAMPs or PAMPs), which are products of a wide range of pathogens (Bent and Mackey, 2007; Chisholm et al., 2006; Jones and Dangl, 2006). The conserved N- and C- termini of bacterial flagellin and the presence of fungal chitin are two well-demonstrated examples of PAMPs for disease and pathogen recognition, but there are many more (Bent and Mackey, 2007; Chisholm et al., 2006; Jones and Dangl, 2006). This recognition and subsequent response is referred to as PAMP-triggered immunity (PTI) and requires Mitogen-activated protein (MAP) kinase signaling cascades that lead to PTI responses via transcriptional modification by the *WRKY* family of transcription factors (Chisholm et al., 2006). The W boxes of *AtWRKY22* and *AtWRKY29* have been shown to be up-regulated as a result of MAP kinase signaling cascades induced by the detection of both chitin and flg22 (a conserved 22-amino acid domain of flagellin) in *Arabidopsis* (Eulgem, 2005; Jones and Dangl, 2006). This flg22 region is essential for flagellin function suggesting that plants evolved a detection system that can't be eluded by

modifications/mutations to PAMPs and encompasses a broad range of detectable pathogens (Anderson et al., 2010; Gomez-Gomez and Boller, 2000; Chinchilla et al., 2006).

Unfortunately, just as plants evolved PTI, pathogens have evolved ways to bypass PTI by suppressing the immunity conferred by PAMP recognition (Chisholm et al., 2006), resulting in enhancement of pathogen virulence and effector-triggered susceptibility (ETS) (Jones and Dangl, 2006). Anderson et al. (2010) proposed that pathogens may cause PTI suppression via some combination of three different mechanisms: detoxification and thus degradation of PTI bioactive products, production of effector molecules that prevent PTI initiation, or those that divert PTI activity. Effector inhibition of PTI is well-documented (Anderson et al., 2010) and the type III secretion system (TTSS) of gram-negative bacterial pathogens is a classic example of an effector delivery system (Chisholm et al., 2006). These types of bacteria can deploy 15-100 effectors (per strain), using TTSS (Bent and Mackey, 2007), that often act as functional mimics of eukaryotic cellular processes to amplify virulence (Jones and Dangl, 2006). To successfully suppress PTI, other effector types may serve structural roles in fungal matrices, promote pathogen dispersal and/or nutrient leakage (Jones and Dangl, 2006).

Yet, as the co-evolution of plant and pathogen continues, plants have acquired the ability to recognize these effector molecules within the cell and, upon recognition, respond with a more targeted and specific counter-attack of effector-triggered immunity (ETI) (Anderson et al., 2010; Chisholm et al., 2006; Kiraly et al., 2007). ETI is the second branch of innate immunity, shares overlap with basal resistance responses (Dangl

and Jones, 2001) and behaves like PTI, only accelerated and amplified so that it confers pathogen resistance (Jones and Dangl, 2006). This effector recognition is accomplished by *R*-gene products, occurs directly or indirectly (Jones and Dangl, 2006), and without this recognition compatible interactions commence leading to host susceptibility (Chisholm et al., 2006). Direct recognition is when an effector, Avr-protein, is recognized and binds to a corresponding *R*-gene product following a gene-for-gene model (Flor, 1971) and, ultimately leading to race/biotype specific ETI and incompatible plant-pathogen interactions (Eulgem, 2005; Jones and Dangl, 2006). One example of a direct R-protein/Avr-protein interaction is found in flax (*Linum usitatissimum*) (Jones and Dangl, 2006). Allelic variants at the flax *L* locus encode for proteins (L5, L6, and L7) that recognize the Avr protein (AvrL567) produced by different flax rust fungus (*Melampsora lini*) pathotypes, eliciting *R*-gene-mediated resistance responses, ETI (Dodds et al., 2006).

Indirect effector detection occurs when *R*-gene products recognize the presence of pathogens by alterations in host effector targets; it's recognition of signals from pathogen-effected cells, referred to as a pathogen-induced modified sense of "self-ness" (Jones and Dangl, 2006; Matzinger, 2002). This is also known as host surveillance (Chisholm et al., 2006) or the "guard hypothesis" (Soosaar et al., 2005; Van der Biezen and Jones, 1998). Here, R-proteins act as guards for specific host proteins (the "guardees") associated with pathogen virulence and when the pathogen causes modifications of the "guardees", like initiation of phosphatase activity, proteolytic cleavage and/or conformational changes (Kiraly et al., 2007), the "guards" (R-proteins)

are alerted to mount resistance responses (Soosaar et al., 2005; Van der Biezen and Jones, 1998). The most well-known example of the guard hypothesis is in *A. thaliana* (Chisholm et al., 2006; Soosaar et al., 2005), where RIN4 (the "guardee") is under surveillance by at least two R-protein guards, including RPM1 and RPS2, and when RIN4 is hyper-phosphorylated due to the presence *Pseudomonas syringae* effectors (AvrRmp1 and AvrB) in the cell, RPM1-mediated resistance responses ensue (Chisholm et al., 2006; Jones and Dangl, 2006). Alternatively, cleavage of RIN4 by a third *P. syringae* effector (AvrRpt2) activates RPS2 and the subsequent RPS2-mediated resistance response (Chisholm et al., 2006; Jones and Dangl, 2006).

There are five classes of R-proteins (Martin et al., 2003) and the majority fall into Class 2 which contain nucleotide-binding site (NB) and leucine rich repeat (LRR) domains (Anderson et al., 2010; Bent and Mackey, 2007), that can recognize diverse kingdoms of biotrophic pathogens (Jones and Dangl, 2006). LRRs are imperfect repeats (Bai et al., 2002) consisting of about 20-30 amino acids and are thought to be involved in both protein-protein (Chisholm et al., 2006) and protein-ligand interactions (Soosaar et al., 2005). NB-LRR proteins tend to be specific to *Avr*-gene product binding and recognition (Belkhadir et al., 2004; Botha et al., 2005) and even though they confer resistance to divergent pathogens, these proteins are fairly similar to each other (Bent, 1996; Soosaar et al., 2005). NB-LRR proteins are subdivided into groups based upon the N-terminal domains: TIR-NB-LRRs have domain homology to Toll-interlueken-1 receptors, CC-NB-LRRs have coiled-coil motifs, and LZ-NB-LRRs show the presence of leucine-zippers (Anderson et al., 2010; Soosaar et al., 2005). Extracellular R-proteins,

eLRRs, also exist and are divided into three sub-classes: RLKs (with cytoplasmic kinase, transmembrane and extracellular LRR domains), RLPs (that share the latter two domains with RLKs, but also have receptor-like proteins), and PGIPs (containing a cell wall LRR and a protein that inhibits polygalacturonase activity) (Chisholm et al., 2006). Over 150 NB-LRR proteins are predicted in *Arabidopsis* and even more in rice (Bai et al., 2002). Evidence to date suggests that conference of pathogen resistance is the sole function of NB-LRR proteins, so plants are evolving numerous genes for employing *R*-gene-mediated ETI (Bai et al., 2002).

The initial *R*-gene-mediated ETI response involves HR with programmed cell death (Soosaar et al., 2005), which seems to be consequential and not causal for this type of resistance (Kiraly et al., 2007). This involves infection-site necrosis in small plant tissues, functions to prevent pathogen growth and can, in resistant tissues, lead to death of the pathogen (Kiraly et al., 2007). HR will generally be confined to infected tissues and may not always be observed during ETI (Jones and Dangl, 2006). When observed, HR will appear as small spots of necrosis perhaps due to ROS activity (Kiraly et al., 2007) on otherwise healthy and normal looking tissue (Figure 1.5 E) (Soosaar et al., 2005). The second response in *R*-gene-mediated, ETI is the deployment of SAR (Soosaar et al., 2005), also known as induced systemic resistance (ISR) (Heil and Bostock, 2002). It is a long-distance, often durable resistance that occurs within hours or days of the initial infection in tissues away from the infection site and creates immunity throughout the plant to subsequent infection by the same or similar pathogens (Eulgem, 2005; Soosaar et al., 2005). SA-production, accumulation (locally, systemically, and in the phloem), and

signal activation of PR proteins are required for the induction of SAR (Dong, 1998; Heil and Bostock, 2002). SAR can also be found with basal resistance responses (Eulgem, 2005). However, as with many of the responses shared between *R*-mediated, ETI resistance and basal resistance, PTI, the difference in resistant outcomes is the quantity and amplification of the response, not necessarily the mechanism of response (Dangl and Jones, 2001; De Ilarduya et al., 2003; Eulgem, 2005).

The last and arguably the most important leg in the arms race of pathogen virulence/avirulence is that natural selection favors pathogens that either modify effectors in avoidance of *R*-gene recognition or increase the number of effectors that can directly suppress ETI (Jones and Dangl, 2006). Then the host *R*-genes would be selected against, new *R*-genes with new specificities would arise, and the race-specific pathogen virulence/avirulence cycle would continue (Anderson et al., 2010; Jones and Dangl, 2006). Therefore, the identification of durable resistance effective against multiple races, strains or biotypes of pests/pathogens would be a significant advantage and could initiate an end to this cycle. This presents an opportunity to explore resistance from a novel perspective, the susceptibility side. Investigations elucidating susceptibility factors are increasing in number and have the potential to be exploited for breeding resistance to various diseases and pests (De Almeida Engler et al., 2005; Pavan et al., 2010). A more detailed description of how susceptibility-based research can lead to enhanced resistance is discussed in Chapter 2.

RWA resistance

It has been suggested that insects, including Hessian fly (*Mayetiola destructor*) and the RWA, engage in a gene-for-gene model of *R*-mediated ETI-like resistance (Botha et al., 2005; Flor, 1971; Miles, 1999; Ohtsuki and Sasaki, 2006). Previously cloned wheat *R*-genes for leaf rust (*Puccinia recondita* f. sp. *tritici*) and cereal cyst nematode (*Heterodera avenae*) contained the NB-LRR domain (Feuillet et al., 2003; Lagudah et al., 1997) and evidence suggests that the *Dn* genes, *R*-genes for RWA resistance, exhibit this motif as well. NB-LRR proteins, functioning as signaling agents and/or ligands for the RWA effector(s), were shown to be regulated during RWA defense responses by Botha et al. (2006). Serine/threonine kinases (STKs) were isolated from RWA-infested wheat tissues, leading to the hypothesis that some *Dn* genes may also be STKs (Botha et al., 2005; Boyko et al., 2006). This was supported by Boyko et al. (2006). Boyko et al. (2006) found the presence of STK-like genes during their cDNA-SSH (suppression subtractive hybridization) profiling study, involving enrichment for differentially expressed wheat transcripts under RWA-infestation.

Neither the exact mechanisms underlying RWA resistance nor the identification of all RWA related R-genes are known or fully understood (Botha et al., 2006; Zhu-Salzman et al., 2004). However, since RWA infestation levels vary among resistant cultivars, it is believed that the genetic background into which the *Dn* resistance gene is bred has a significant impact on the gene's effectiveness (Botha et al., 2005). Despite lack of information, this predicted *R*-gene meditated, gene-for-gene interaction would mean, as it does with other pathogens, that if a RWA biotype were to lack, modify, or multiply their

Avr-genes, a resistant cultivar could be rendered susceptible and suffer the consequences of compatible RWA-host interactions (Flor, 1971; Jones and Dangl, 2006). To date, 12 named *Dn* resistance genes have been identified, however, few afford resistance to RWA biotype 2 which reinforces the need for more durable resistance (Collins et al., 2005a; Haley et al., 2004; Peng et al., 2007; Smith et al., 2004).

Compatible and incompatible RWA-host interactions

When *R*-gene-mediated, ETI is conferred under RWA-infestation, incompatible or resistant interactions ensue (Heil and Bostock, 2002). The consequences of these incompatible interactions fall into three categories: antibiosis, antixenosis, and tolerance (Painter, 1958; Smith, 2005). Antibiosis, the most common category (Unger and Quisenberry, 1997), is a measure of aphid fecundity, affecting the ability of RWA to grow, develop and reproduce under adverse biological conditions (Painter, 1958). Aphids feeding on antibiotic plants have fewer progeny, reduced rates of reproduction, and experience a decrease in their life spans (Botha et al., 2005; Unger and Quisenberry, 1997; Voothuluru et al., 2006). These effects may be due to defensive aphid-deterrent chemical production from the plant (allelochemicals) or a lack of sufficient nutrients for aphid growth and/or reproduction (Lazzari et al., 2009). Antixenosis, or non-preference, occurs when the host plant is not an acceptable food source, shelter or ovipositional area for aphids and given the chance, they would migrate to a more optimal host (Painter, 1958; Smith et al., 1992). Antibiosis and antixenosis may also be dependent on the aphids' ability to select appropriate feeding sites, the ease with which they can access the phloem, and the properties of the sieve elements (Lazzari et al., 2009). Phases of aphid

feeding have been investigated by employing the use of electronic penetration graphs (EPGs) and results have found that aphids took up to four times as long to find the phloem, salivated more and ingested less on resistant and non-host plants than on their susceptible host counterparts (Girma et al., 1992). However, using antibiosis or antixenosis as RWA management strategies impose strong selection pressure on the evolution of new RWA biotypes that can overcome these adverse interactions (Haile et al., 1999; Hawley et al., 2003).

Unlike antibiosis and antixenosis, tolerance is resistance based on the ability of the plant to weather the storm. It involves survival under heavy levels of infestation and allows for the increased production of dry plant matter compared to that of susceptible genotypes (Painter, 1958; Voothuluru et al., 2006). Tolerance is typically analyzed through a measure of reduction of infested plant height and fresh and dry weight (above- and below-ground) as they compare to un-infested controls (Dixon et al., 1990; Voothuluru et al., 2006). Since plant tolerance as a resistance category does not place selective pressure on emergence of new biotypes, it has been the recipient of increased attention in resistant cultivar development over either antibiosis or antixenosis (Haile et al., 1999). Additionally, a tolerant plant decreases the level of economic loss due to RWA damage and may delay the use of chemical treatment, making it desirable for use in breeding programs (Lazzari et al., 2009).

Ultimately, breeding for RWA resistance has resulted in cultivars with components of various levels of each of the three categories (Franzen et al., 2007); a well-rounded combination for more effective resistance. Therefore, almost every study reporting on

the nature of inheritance and characterization of new resistance genes and/or cultivars does so by assessing antibiosis, antixenosis, and tolerance.

In the absence of specific *R*-genes, mutation of *Avr*-proteins (effectors), or the deployment of multiple effectors [all resulting in suppression or avoidance of ETI (Jones and Dangl, 2006)], compatible RWA-host interactions commence (Heil and Bostock, 2002). Compatible or susceptible RWA-host interactions cause a series of phenotypic symptoms including chlorosis and longitudinal streaking, leaf rolling, stunted growth and head trapping (Figure 1.5); all of which lead to significant reductions in yield and often death (Botha et al., 2006; Burd et al., 1998; Franzen et al., 2008; Smith et al., 1992). Evidence has suggested that RWA feeding alters chlorophyll fluorescence, while decreasing chlorophyll *a*, *b*, chlorophyllide, and carotenoids in wounded areas of the plant (Burd and Elliott, 1996; Ni et al., 2002; Wang et al., 2004). Burd and Elliot (1996) reported that susceptible RWA-infested cultivars TAM W-101, Pavon, and Wintermalt showed significant reductions in total chlorophyll content (and the individual *a*, and *b* components), compared to their non-infested controls (~30, 40, and 48%, respectively). They also found that resistant cultivars exhibited no significant loss of chlorophyll when infested with RWA (Burd and Elliott, 1996).

Chlorosis resulting from RWA feeding has been shown to occur by-way of chlorophyll catabolism different from that of natural senescence, but not as well understood (Ni et al., 2002). Natural senescence-related chlorophyll loss arises from involvement of the pheophorbide *a* and oxidative bleaching pathways (Ni et al., 2001). RWA-induced chlorosis has been attributed only to the former, with no evidence of

oxidative bleaching activity (Ni et al., 2002; Wang et al., 2004). RWA-infested 'Tugela' and 'Arapahoe', showed high levels of Mg-dechelatase activity and low levels of chlorophyllase, both major components of the pheophorbide *a* pathway (Figure 1.6), compared to infestation with non-chlorosis-inducing Bird cherry-oat aphids (*Rhopalosiphum padi* L.) (Ni et al., 2002; Wang et al., 2004). This indicates that in these susceptible cultivars, chlorosis due to chlorophyll degradation is being caused by limited production of chlorophyllide *a* (Wang et al., 2004). Since chlorophyll synthase can reuse chlorophyllide *a* to produce chlorophyll *a*, reduced availability of chlorophyllide *a* tips the scale towards chlorophyll degradation instead of biosynthesis (Figure 1.6) (Wang et al., 2004).

Reduced photosynthetic rates and chlorophyll fluorescence, and their roles in aphid-induced chlorosis have been attributed to two mechanisms (Haile et al., 1999): inhibition of photosynthetic electron transport (Burd and Elliott, 1996; Miller et al., 1994b), and/or chemical disintegration of chloroplasts (Fouche et al., 1984). However, additional research has indicated that they may be the result of high levels of photochemical fluorescence quenching and gas exchange responses effected by aphid feeding and that damage or modification of chlorophyll content may be a secondary consequence (Franzen et al., 2007, 2008). Interestingly, upon aphid removal, tolerant cultivars show complete recovery of photosynthetic capacity whereas susceptible cultivars do not (Haile et al., 1999). Chlorosis is very damaging to the health of the host plant, its ability to generate sufficient nutrients for growth and seed production, and can be responsible for yield reductions of up to 50% (Smith et al., 1992).

Leaf rolling, referred to as pseudo-galling (Burd et al., 1993), is a very important aspect of RWA feeding on susceptible plants because it provides protection for the aphids, acting as a sanctuary from the environment and pest management strategies like bio-control and insecticides (Haile et al., 1999; Voothuluru et al., 2006). It's an early-occurring symptom that can affect both mature and newly formed leaves (Botha et al., 2005), but the mechanisms behind this phenotype are lacking since most studies of leaf function focus on photosynthesis related investigations (Van Volkenburgh, 1999). Exposure to external biotic and abiotic stresses can lead to unbalanced cell expansion in mature leaves (reviewed by Botha et al., 2005). The mechanism coordinating leaf expansion is regulation of ion [mainly potassium (K^+)] distribution across the plasma membrane (Van Volkenburgh, 1999). It has been suggested that these ion gradients are disrupted and/or the plasma membrane suffers altered permeability from aphid feeding (Moran et al., 2002), resulting in K^+ and H_2O leaving the upper cells (sites of aphid salivary entry and/or wound signaling) and entering the lower cells (Van Volkenburgh, 1999). This would cause rapid cell expansion and turgor in the lower cells, upper cell collapse, and subsequent mature leaf rolling (reviewed by Botha et al., 2005; Van Volkenburgh, 1999).

When convoluted rolling prevents the unfurling of new leaves, it negatively impacts plant growth accumulation by stunting the development of new leaves (Burd et al., 1998). Leaf unfolding and expansion in grasses depends on bulliform and mesophyll cell enlargement by the meristem at the base of the developing leaf and this has been tightly correlated with maintenance of leaf turgor (Burd and Burton, 1992). Burd et al. (1993)

reported that susceptible wheat cultivars Beagle 82 and TAM W-101 exhibited significantly lower (more negative) leaf-water potential and osmotic potentials that were either unchanged or significantly higher (less negative). This suggests that RWA-infested susceptible cultivars have an inability to osmotically adjust to decreased leaf-water content, resulting in loss of leaf turgor (Burd et al., 1993). Loss of turgor below minimum thresholds for elongation and extensibility of cells culminates in the prevention of the unfolding of new leaves (Burd and Burton, 1992). Additionally, leaf rolling minimizes photosynthetic leaf area, impacting total plant sugar production (Macedo et al., 2003). Unlike photosynthetic capacity and carbon assimilation that recover upon the removal of RWA, convolutedly rolled leaves will not; leaves will remain rolled and new growth trapped (Burd and Burton, 1992). Since leaf rolling is closely linked to the biological fitness of RWA, it is important to deploy resistant cultivars that can maintain flat leaves and thus inhibit the RWA from creating protective, defensible habitats (Burd et al., 1993).

Aphid feeding on the flag leaf results in a distorted head, referred to as head trapping, which negatively impacts self-pollination and, most importantly, grain filling (Smith et al., 1992). In fact, under heavy infestation where death is avoided, grain weights can be reduced up to 80% (Voothuluru et al., 2006). The combination of all these RWA symptoms and their destructive impact on the host plant results in the reduction of yield and quality of seed, creating a major problem for farmers and total wheat production in the western United States. The degree to which yields are negatively impacted by RWA infestations depends on factors such as infestation levels (migratory and, often more

importantly, overwintering), precipitation, temperature, field characteristics (soil pH, other pathogens, etc), geographic region, plant growth-stage, plant health, and choice of cultivar (Merrill et al., 2009; Mirik et al., 2009; Randolph et al., 2007, 2008). Since 1987, over $1 billion in economic losses due to the RWA have accrued with 60% of that reported in southeastern Colorado, western Kansas, southwestern Nebraska, and the panhandles of Texas and Oklahoma (Smith et al., 2004). When RWA are abundant, they can greatly damage wheat and barley in a very short period of time (Mirik et al., 2009), resulting in 100% yield reductions (Elliot et al., 2007).

The challenge for increasing wheat yields lies not only with the understanding of a genome sequence (yet to come) but also with considering how breeders will be able to stably integrate this information into adapted high performing cultivars. It will take an integrated effort among wheat scientists; breeders, geneticists, pathologists, physiologists and more, to address the challenge facing agricultural production and food security.

FIGURES

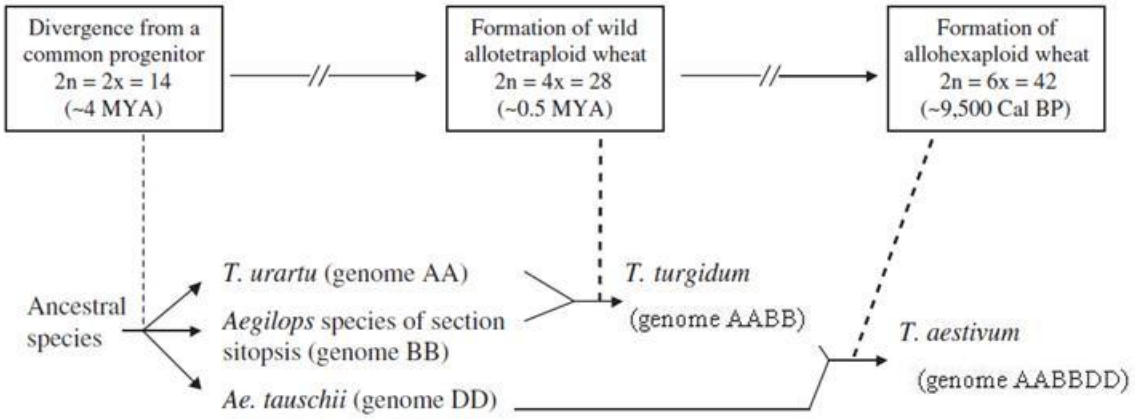

Figure 1.1 The proposed origin of hexaploid bread wheat from the hybridization events of diploid and tetraploid progenitors [adapted from Feldman and Levy (2005)].

Figure 1.2 The current region of N. American RWA infestation [reproduced from Hodgson and Karren (2008)].

Figure 1.3 Russian wheat aphids on wheat and in the process of giving birth to live young.

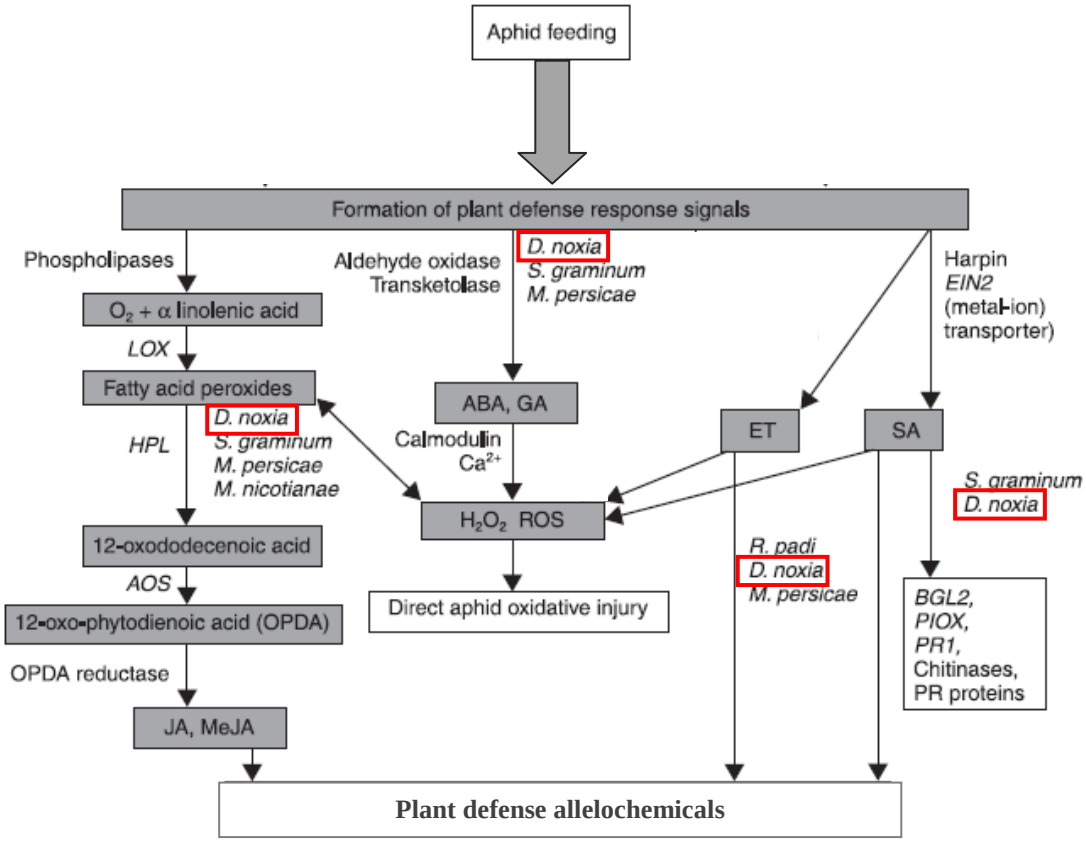

Figure 1.4 A summary of host defense signaling pathways triggered in response to aphid feeding [adapted from Smith and Boyko (2007)].

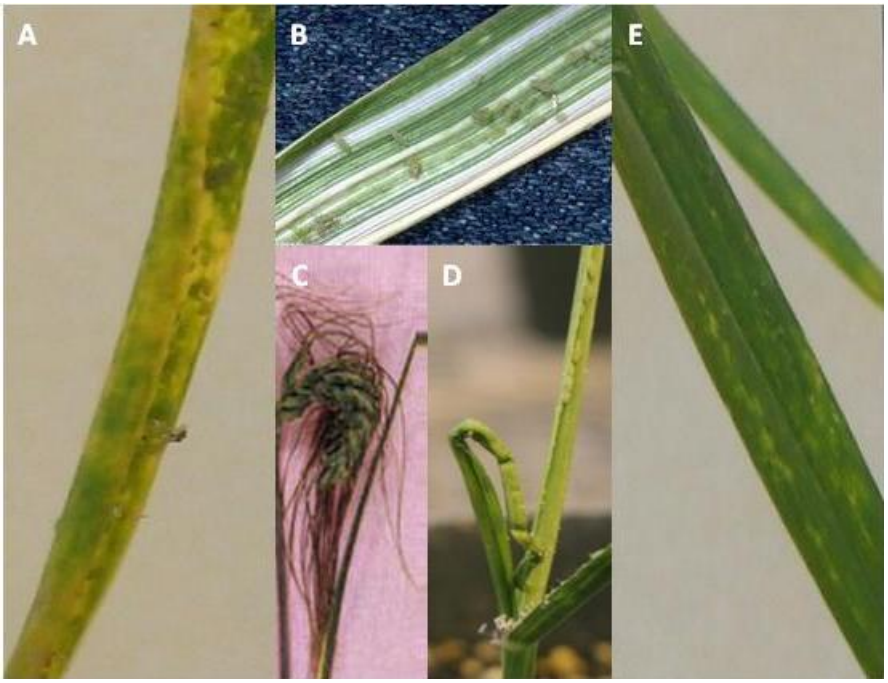

Figure 1.5 Symptoms of RWA feeding: **A** chlorosis, **B** longitudinal streaking, **C** head trapping, **D** mature leaf rolling and unfurling prevention in new growth, and **E** HR lesions on a resistance leaf [Photos **B** and **C** taken from Hodgson and Karren (2008)].

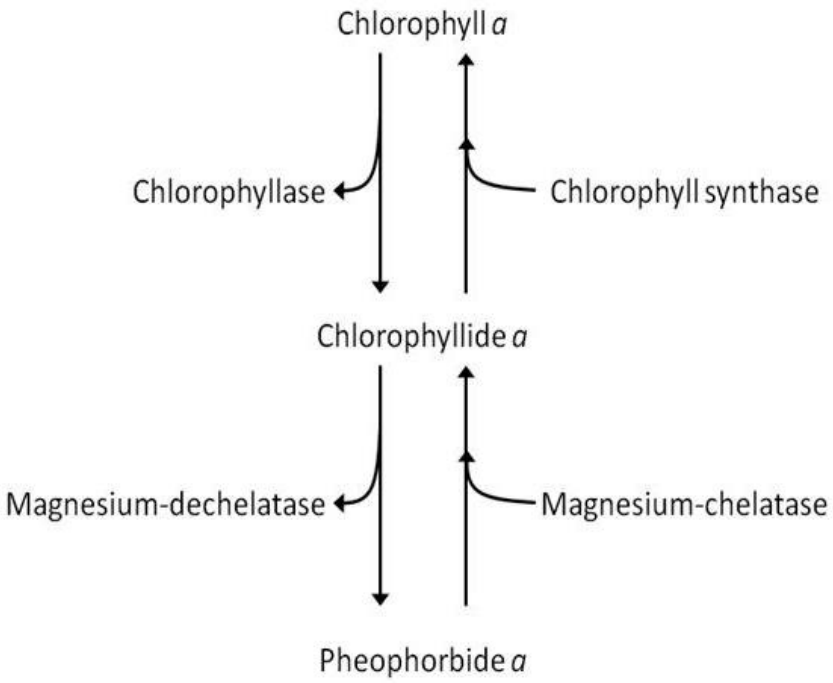

Figure 1.6 The chlorophyll *a* degradation (downstream) and biosynthesis (upstream) pathways as observed in hexaploid wheat [adapted from Wang et al. (2004)].

OBJECTIVES AND GOALS

The overall goal of this study was to identify novel sources of RWA resistance that could be utilized in wheat breeding programs for the production and deployment of cultivars containing enhanced RWA resistance. To address this goal, aspects from both sides of the resistance coin were explored: investigation and characterization of a new *R*-gene for conference of *R*-gene-mediated resistance and reduced expression of a gene predicted to be involved in the susceptible or compatible RWA-wheat interaction to potentially induce resistance in a previously susceptible genotype. The former was achieved through molecular marker mapping of resistance in an Iranian wheat landrace accession by incorporating bulked segregant analysis and high-throughput genotyping technology. Polymorphic microsatellite markers were used in conjunction with phenotyping in an $F_{2:3}$ population to identify the chromosomal location of resistance. To address the latter, virus-induced gene silencing (VIGS) was utilized to assess the potential involvement of a gene previously shown to be up-regulated in compatible RWA-wheat interactions on aphid reproduction and host symptom development. Whether resistance could be conferred to a susceptible wheat genotype upon reduction of transcript abundance was assessed. By exploring resistance from the perspective of plant resistance mechanisms, as well as host compliance with functions essential to aphid fitness, the prospect of durable RWA resistance may come closer to reality.

CHAPTER TWO

VIRUS-INDUCED GENE SILENCING FOR RUSSIAN WHEAT APHID RESISTANCE

ABSTRACT

Russian wheat aphid (RWA), *Diuraphis noxia* (Kurdjumov), is a significant insect pest of wheat (*Triticum aestivum* L.) and has had a major economic impact worldwide, especially on winter wheat in the western part of the United States. The continuing emergence of new RWA biotypes virulent to existing resistance genes reinforces the need for the discovery of more durable resistance. Inhibition of essential products for pest/pathogen growth and development via knockdown of genes involved in host plant susceptibility could produce resistance in previously susceptible plants. The objective of this study was to test whether silencing a candidate gene potentially involved in compatible interactions between RWA and wheat would confer resistance to a susceptible wheat genotype. Several genes were identified as differentially expressed between the susceptible cultivar, 'Gamtoos-S' (GS), and the near-isogenic resistant line, 'Gamtoos-R' (GR; carrying *Dn7*), in a previous transcript profiling study. The goals of

the current research were to: 1) identify a candidate gene up-regulated in compatible RWA-wheat interactions (GS compared to GR), 2) determine whether the gene is involved in these compatible interactions by assessing the effects of gene silencing on host symptom development and aphid reproduction and 3) determine whether RWA resistance may be conferred to a susceptible wheat cultivar by silencing. Barley stripe mosaic virus (BSMV)-mediated virus-induced gene silencing (VIGS) was employed to test whether *(1,3;1,4)*-β-*glucanase* is involved in the susceptible reaction of GS. Controlled infestation with RWA biotype 2 was used to assess aphid reproduction and host symptom development. *(1,3;1,4)*-β-*glucanase* transcript abundance in the silenced treatment was reduced to levels similar to GR (P=0.600). Aphids on the silenced treatment reproduced less per day (P<0.0001) and had longer pre-nymphipositional periods than those on GS (P=0.003). Compared to GS, the silenced treatment exhibited less chlorosis (P<0.0001), greater dry weight (P=0.044), and had lower aphid to dry weight ratios (P=0.039). However, aphid-induced leaf rolling was unaffected by reduction of *(1,3;1,4)*-β-*glucanase* expression (P=1.000), suggesting separate mechanisms for leaf rolling and chlorosis. Aphid reproduction and host symptom development had linear relationships with *(1,3;1,4)*-β-*glucanase* transcript levels. VIGS construct sequence analyses indicated the possibility of non-target silencing of *(1,3)*-β-*glucanase*, but how transcript abundance of *(1,3)*-β-*glucanase* was altered by the VIGS construct designed for this study has yet to be determined. Our results suggests that *(1,3;1,4)*-β-*glucanase* may be a susceptibility factor that could be exploited as a potential avenue for aphid resistance. Subsequent investigation into the possible co-silencing of *(1,3)*-β-*glucanase* and a more detailed look into the role of *(1,3;1,4)*-β-*glucanase* in

RWA susceptibility will provide insight into how *(1,3;1,4)*-β-*glucanase* suppression can be used in the implementation of novel RWA resistance in wheat breeding programs.

INTRODUCTION

The Russian wheat aphid (RWA), *Diuraphis noxia* (Kurdjumov), is an important insect pest of wheat (*Triticum aestivum* L.) and barley (*Hordeum vulgare* L.) and has had a major economic impact worldwide, especially on winter wheat in the western part of the United States (Burd et al., 2006; Haley et al., 2004; Weiland et al., 2008). Direct and indirect damage due to RWA in the western U.S. alone exceeded $800 million within the first seven years after its introduction in 1986 (Morrison and Peairs, 1998). RWA feed on sap located in the phloem of their host plants via intercellular insertion of the stylet into plant sieve tubes, although some cellular penetration may occur (Miles, 1999; Will and Van Bel, 2006). The aphid injects an eliciting agent, shown to be in the protein fraction (Lapitan et al., 2007a), that induces defense/resistance responses in resistant plants or symptom development in susceptible plants (Smith and Boyko, 2007). Compatible RWA-host interactions induce a series of phenotypes including chlorosis and longitudinal streaking, leaf rolling, head trapping and stunted growth; all of which lead to significant reductions in yield and plant death under heavy infestations (Botha et al., 2006; Burd et al., 1998; Franzen et al., 2008; Smith et al., 1992).

The effects of RWA feeding on plants are unlike that of most chewing insects in that they closely resemble disease infection (Halbert and Stoetzel, 1998). It has been suggested that the RWA-host interaction follows an *R*-gene-mediated, gene-for-gene model creating race/biotype-specific resistance (Flor, 1971; Miles, 1999; Ohtsuki and Sasaki, 2006). To date, 12 named resistance genes have been identified; however, few afford resistance to the most virulent biotype, RWA biotype 2, reinforcing the need for

more durable forms of resistance (Collins et al., 2005a; Haley et al., 2004; Peng et al., 2007; Smith et al., 2004). Genes regulating resistance have been studied extensively for many years, whereas the genetic mechanisms controlling susceptibility or compatible interactions have received limited attention (Lorang et al., 2007; Pavan et al., 2010; Vogel et al., 2002). Investigations elucidating susceptibility factors are increasing in number, however, and have the potential to be exploited for breeding resistance to various diseases and pests (De Almeida Engler et al., 2005; Pavan et al., 2010).

Susceptibility factors fall into one of two categories: negative regulators or susceptibility genes (De Almeida Engler et al., 2005; Pavan et al., 2010). To induce susceptible interactions, pests/pathogens must bypass or overcome plant defenses and it has been suggested that suppression of plant innate immunity by host protein (effector target) interactions with pest/pathogen effectors results in such effector-triggered susceptibility (Chisholm et al., 2006; Nomura et al., 2005; Pavan et al., 2010). This supports the hypothesis that silencing the host protein (effector target) could lead to resistance by the removal of defense suppression (Giordanengo et al., 2010; Miles, 1999; Pavan et al., 2010). Resistance to powdery mildew in barley (causal agent *Blumeria graminis* f.sp. *hordei*) conferred by removal of defense suppression from loss-of-function mutations of the *Mlo* gene, is a classic example of negative regulation (Buschges et al., 1997; Humphry et al., 2006). Several other examples exist including zinc binding proteins (*RAR1*), *WRKY* transcription factors, and mitogen-activated protein kinases (*EDR1* and *MPK4*) in *Arabidopsis* (Buschges et al., 1997; reviewed by Pavan et al., 2010).

Susceptibility genes (*S*-genes) tend to be dominant genes that, upon recognition of the corresponding virulence or effector gene products, elicit compatible interactions in an inverse gene-for-gene system and can confer recessive resistance upon functional mutation (Friesen et al., 2008; Lorang et al., 2007; Pavan et al., 2010). This type of resistance was first identified through studies of *Arabidopsis* mutants that prevented powdery mildew establishment and growth (De Almeida Engler et al., 2005). Loss-of-function mutations in the *PRM6*, a pectate lyase-like gene, exhibited strong resistance to powdery mildew independent of host defense activation, cell death or presence of a resistance gene, suggesting that this type of resistance comes about via denial of pathogen growth and developmental requirements from the host (Vogel et al., 2002). *S*-genes have been identified in wheat, mostly from its compatible interactions with tan spot (*Pyrenophora tritici-repentis*) (Faris and Friesen, 2005; Friesen et al., 2003; Friesen and Faris, 2004) and leaf blotch (*Stagonospora nodorum*) (Friesen et al., 2008).

While investigations into susceptibility factors for plant-pathogen interactions are growing in number, such reports involving insect interactions have been restricted mainly to whole genome transcript profiling studies comparing expression of resistant and susceptible cultivars under infested conditions (Botha et al., 2010; Wang et al., 2008). Inhibition of essential products for pest/pathogen growth and development (*e.g.* appropriate nutrients or ease of access to phloem, in the case of aphids) via knockdown of *S*-genes and/or other genes involved in host plant susceptibility could produce resistance in previously susceptible plants (Pavan et al., 2010; Vogel et al., 2002). Therefore, investigation into compatibility genes involved in plant-insect interactions is warranted.

Virus-induced gene silencing (VIGS) is a post-transcriptional gene silencing (PTGS) technique that takes advantage of the dsRNA-mediated antiviral defense mechanism in plants (Baulcombe, 1999; Fu et al., 2007; Shao et al., 2008). VIGS has been used to study gene function in both dicots and monocots (Burch-Smith et al., 2004; Holzberg et al., 2002; Scofield and Nelson, 2009). The application of VIGS employing barley stripe mosaic virus (BSMV) as a vector has mainly been a reverse genetic tool to investigate plant disease defense responses (Cakir et al., 2010; Dagdas et al., 2009; Scofield et al., 2005) and has recently been shown to be effective for examining insect resistance as well. Van Eck et al. (2010) used BSMV-mediated VIGS for examination of insect resistance in wheat and showed that wheat plants silenced for *WRKY53* had decreased resistance to RWA.

The objective of this study was to test whether silencing a candidate gene predicted to be involved in compatible interactions between RWA and wheat would confer resistance to a susceptible wheat genotype. Botha et al. (2010) recently compared the expression profiles of two near-isogenic lines (NILs) of wheat during infestation with RWA. Several genes were identified as differentially expressed between the susceptible line, 'Gamtoos-S' (GS), and the resistant line, 'Gamtoos-R' (GR; carrying the *Dn7* resistance gene). The goals of the current research were to: 1) identify a candidate gene up-regulated in compatible RWA-wheat interactions (GS compared to GR), 2) determine whether the gene is involved in these compatible interactions by assessing the effects of

gene silencing on host symptom development and aphid reproduction and 3) determine whether increased RWA resistance may be conferred to a susceptible wheat cultivar by silencing.

RESULTS

Selection of (1,3;1,4)-β-glucanase as a candidate gene

Based on the microarray conducted by Botha et al. (2010), several genes were differentially expressed between RWA biotype 2 infested GS and GR. Differential expression data from that microarray were examined and a list consisting of 15 potential candidate genes was produced based on logfold expression change in GS/GR at 5 hours post-infestation (hpi) (Table 2.1). Transcripts ranked 1st (Ta.10.2.S1_x_at) and 8th (Ta.10.1.S1_a_at) on this list corresponded to wheat *(1,3;1,4)*-β-*glucanase* sequences (accessions Z22873.1 and Z22874.1, respectively) (Lai et al., 1993). It is unclear whether these transcripts correspond to the same gene, are allelic variants at the same locus or are homoeologs present on different chromosomes and/or genomes (Lai et al., 1993). Based on rank, *(1,3;1,4)*-β-*glucanase* was chosen as the candidate gene for this study. Increased *(1,3;1,4)*-β-*glucanase* transcript abundance in GS compared to GR at 5 hpi with RWA biotype 2 was verified by examining the differential expression of *(1,3;1,4)*-β-*glucanase* in cDNA of GS and GR (5 hpi) using real-time quantitative PCR (RT-qPCR). The melt curve from the RT-qPCR reactions consisted of a single peak (Figure 2.1), which verified that the RT-qPCR primers used in this study amplify only *(1,3;1,4)*-β-*glucanase* in GR and GS cDNA. Under non-infested conditions, *(1,3;1,4)*-β-*glucanase* transcript abundance in GR is approximately 66% of the level observed in GS (Figure 2.2). However, once RWA biotype 2 infestation begins, the degree of differential expression increases. Expression of *(1,3;1,4)*-β-*glucanase* in GR at 5 hpi is about 97% less than GS (Figure 2.2). This differential expression at 5 hpi, verified the results of the Botha et al. (2010) study.

Sequence analysis reveals potential non-target silencing

A VIGS construct was designed from a region of wheat *(1,3;1,4)*-β-*glucanase* spanning the end of the coding region into the 3' UTR. To identify potential non-targets of silencing, the VIGS construct sequence was subjected to BLASTn pair-wise analyses comparing word sizes ≥ 11 bases with published sequences that resembled the construct (Zhang et al., 2000). Published sequence information about the wheat genome is lagging behind that available for other species such as rice (*Oryza sativa* L.), maize (*Zea mays* L.), *Arabidopsis thaliana* L. and barley with smaller, less complex genomes (reviewed by Wanjugi et al., 2009; Scofield and Nelson, 2009). As such, comparative analysis is a valuable tool for wheat researches to gain insight into the potential composition of the wheat genome by comparing wheat sequences to those found in other genomes. Subjecting the VIGS construct designed from wheat *(1,3;1,4)*-β-*glucanases* to an across-species BLASTn search, helps identify potential non-targets of silencing for which we have no published sequence information in wheat. The 56 sequences that most resembled the VIGS construct based on the BLASTn search are listed in Table 2.2, sorted by maximum identity. Of these, 87.5% were in the Poaceae family and all 56 consisted of *(1,3;1,4)*-β-*glucanases*, *(1,3)*-β-*glucanases*, full insert clones, hypothetical proteins and one complete genome sequence (Table 2.2). Alternative names for glucanases include glucosidases and glucan endohydrolases; lichenases are analogous to *(1,3;1,4)*-β-*glucanases* (Gasteiger et al., 2003). These results suggested that *(1,3)*-β-*glucanases* were the most likely non-targets for silencing using the VIGS construct designed for this study.

Since the objective of this study was to silence *(1,3;1,4)*-β-*glucanases* in wheat, a detailed alignment of the wheat *(1,3;1,4)*-β-*glucanase* and *(1,3)*-β-*glucanase* sequences was done using Geneious Pro™ v5.0.3, followed by an extraction of the VIGS region for analysis. The alignment was a global alignment with free end gaps and was constructed using the following parameters: cost matrix of 65% (5.0/-4.0), gap open penalty of 12, gap entension penalty of 3, and two refinement iterations (Figure 2.3). Results of the alignment revealed several small regions of shared nucleotide identity between the *(1,3)*-β-*glucanases* and the VIGS construct, in addition to two major regions that could possibly trigger silencing of *(1,3)*-β-*glucanase*s upon cleavage by the dicer-like complex (Figure 2.3; Figure 2.4). These 56 bp and 47 bp regions share high pair-wise identities with wheat *(1,3)*-β-*glucanases* (78.6% and 87.2%, respectively) (Table 2.3). Within the 47 bp region, there is a section of 23 consecutive bases of perfect identity between the construct and the *(1,3)*-β-*glucanases* sequences and could result in siRNAs that would trigger silencing of this non-target (Figure 2.4). Therefore, it was concluded that *(1,3)*-β-*glucanases* are a non-target that could have potentially been silenced with the VIGS construct used in this study.

(1,3;1,4)-β-glucanase expression reduction resulting from virus-induced gene silencing

Virus-induced gene silencing of *(1,3;1,4)*-β-*glucanase* in GS was conducted to assay effects on aphid reproduction and plant symptom development during RWA biotype 2 infestation. Relative comparison of transcript abundance obtained by RT-qPCR was used to determine the amount of *(1,3;1,4)*-β-*glucanase* silencing achieved by the VIGS contruct designed for this study, as well as *(1,3;1,4)*-β-*glucanase* expression in the

control treatments. By employing the use of BSMV-mediated VIGS, mean expression of *(1,3;1,4)*-β-*glucanase* was reduced by 60.8%, a level similar to that of the GR treatment (*P*=0.600), relative to the mean transcript abundance exhibited in the GS control (Figure 2.5). Additionally, the viral control GS+BSMV$_{BG-}$ plants exhibited *(1,3;1,4)*-β-*glucanase* transcript levels similar to the susceptible GS checks (*P*=0.945).

Transcript levels affect RWA fitness and reproductive ability

To determine the effect of transcript levels on RWA reproduction, nymph production was recorded. Nymphs born to the foundress in each cage were counted and removed daily to estimate effects of silencing on antibiosis. The average number of nymphs born per day was averaged across four biological repeats within each treatment (Table 2.5). There were 0.92 nymphs day^{-1} on GS+BSMV$_{BG+}$ plants, significantly less than the 1.46 nymphs day^{-1} on GS (*P*<0.0001) (Table 2.5); however, the mean of the silenced treatment was not reduced to the 0.65 nymphs day^{-1} measured on GR (*P*=0.046) (Table 2.5).

Aphid to plant biomass ratios were calculated and averaged across the four biological replications per treatment which allowed us to observe antixenotic and/or antibiotic effects of transcript levels, while taking into account any reduction in feeding surface area due to aphid-induced inhibition of plant growth (Figure 2.6). GR plants had the lowest ratio of RWA to dry weight (512 aphids g^{-1}). The silenced treatment exhibited a ratio of aphids to gram of dry weight (4283 aphids g^{-1}) significantly lower (*P*=0.039) than the susceptible check, GS (8280 aphids g^{-1}) (Figure 2.6).

Pre-nymphipositional period (PNP), the number of days from the birth of the foundress to the start of her reproduction, was recorded for each of the four treatments (Table 2.5). The start of reproduction took an average of 9.0 days in the silenced treatment which was significantly longer than the 7.3 days the foundresses took on GS (P=0.003) (Table 2.5). The nature of this delay in reproduction was not investigated in this study; however these PNP values were consistent with previous findings that adults are generated in ~7-10 days (Hodgson and Karren, 2008). The viral control was similar to GS for each of the three RWA reproductive measurements (average births per day P=0.097, RWA to dry weight ratio P=0.526, and PNP P=0.317) (Table 2.5).

With respect to aphid reproduction, the silenced treatment exhibited increased resistance over the susceptible controls through reduced aphid reproduction. Compared to GS, the GS+$BSMV_{BG+}$ treatment supported fewer aphid births per day, promoted longer pre-reproductive periods and had fewer aphids per gram of dry plant weight during uncaged infestation.

Reduced levels of expression improve plant phenotype during RWA stress

The GS+$BSMV_{BG+}$ treatment was visually more vigorous and less chlorotic than the other GS treatments (Figure 2.7), so chlorosis and leaf rolling scores, along with dry weight measurements were recorded to quantify these visual observations (Table 2.5; Table 2.6). Chlorosis and leaf rolling was assessed on scales of 1-9 and 1-4, respectively, using a system modified from Collins et al. (2005). The GS+$BSMV_{BG+}$ treatment with *(1,3;1,4)*-β-*glucanase* transcript levels similar to the resistant check, GR, showed a significant reduction in chlorosis from that of the susceptible check GS, from 7.0 to 2.5

(P<0.0001), with scores similar to GR (2.0) (P=0.521) (Table 2.5). Additionally, the presence of the BSMV vector alone had no significant effect on chlorosis as seen by the lack of difference in chlorosis between GS and GS+$BSMV_{BG-}$ (score of 6.8) (P=0.747). Leaf rolling was unaffected by transcript reduction as there was no difference in leaf rolling scores among the GS backgrounds (scores of 4.0) which were much more tightly rolled than GR (leaf rolling of 1.0) (P=1.000) (Table 2.5).

To assess effects of silencing and aphid feeding on plant growth, dry weight measurements, both above- and below-ground, were recorded (Table 2.6). The GS+$BSMV_{BG+}$ treatment weighed less than GR (0.645 g and 1.193 g, respectively), however, they weighed more than the 0.297 g of GS (P=0.044) and the viral controls, $BSMV_{BG-}$ (P=0.044) (Table 2.6). Similar trends were observed with the above-ground biomass, but silencing did not have a significant impact on dry root weight, compared to GS (P=0.094) (Table 2.6).

(1,3;1,4)-β-glucanase transcript levels show linear relationship with both phenotype and aphid reproduction

To determine whether there was a linear relationship between levels of *(1,3;1,4)*-β-*glucanase* expression at 14 days post-RWA infestation (19 days post-silencing) and the RWA reproduction and plant phenotype data, regression analyses were performed (Figure 2.8). The strongest linear relationships were observed with pre-nymphipositional period (R^2=0.723, P<0.0001) and average number of RWA births per day (R^2=0.814, P<0.0001), the former having an inverse relationship with transcript abundance (Figure 2.8, B and A, respectively). However, significant relationships were also observed

between *(1,3;1,4)*-β-*glucanase* transcript abundance and the aphid to dry weight ratio (R^2=0.553, *P*=0.0004), chlorosis (R^2=0.600, *P*=0.0002), and total dry plant weight (R^2=0.494, *P*=0.0011), the latter showing an inverse relationship (Figure 2.8, C-E).

DISCUSSION

The presence of certain host genes is required for induction of susceptible plant-pest/pathogen interactions, which supports the hypothesis that resistance could be conferred via knockdown of these genes (De Almeida Engler et al., 2005; Pavan et al., 2010). These susceptibility factors function as negative regulators of plant defense mechanisms, or as *S*-genes required for growth and development of the pest/pathogen (De Almeida Engler et al., 2005; Pavan et al., 2010). We investigated whether knockdown of *(1,3;1,4)*-β-*glucanase* transcript expression in a susceptible genotype, using BSMV-mediated VIGS, would correspond to increased resistance to RWA by assessing aphid reproduction and host symptom development.

Wheat (1,3;1,4)-β-glucanase is related to EI, one of the two barley (1,3;1,4)-β-glucanase isoenzymes. The EI and EII isoenzymes differ by 25 amino acid substitutions and wheat (1,3;1,4)-β-glucanase shares 22 of the 25 substitutions with EI (Lai et al., 1993; Slakeski and Fincher, 1992a; Walti et al., 2002). EII, found in the scutellum and aleurone of germinated grain, has yet to be identified in leaf tissues of wheat and is reported to be germination-specific, while EI is expressed in vegetative tissues such as roots and leaves (McFadden et al., 1988; Roulin and Feller, 2001; Slakeski and Fincher, 1992a,1992b). EI expression increases significantly during extended exposure to darkness due to decreased leaf sugar levels (Roulin et al., 2002; Roulin and Feller, 2001). The role that phloem feeding, sugar-depleting aphids play in this relationship is not known, although an increase in wheat *(1,3;1,4)*-β-*glucanase* expression under RWA infestation has been shown (Botha et al., 2010). *Gsn1*, the *(1,3;1,4)*-β-*glucanase* rice

homolog with 82% amino acid similarity to EI, is induced by fungal elicitors, salicylic acid, ethylene and mechanical wounding, suggesting a stress-inducible function not related to development or germination (Hao et al., 2009; Nishizawa et al., 2003; Simmons et al., 1992). This function in rice may also be plausible for barley EI and wheat *(1,3;1,4)*-β-*glucanase* expression in leaves, since their function in vegetative tissues is not fully understood (Akiyama et al., 2009; Roulin et al., 2002).

Average *(1,3;1,4)*-β-*glucanase* silencing levels in the GS+BSMV$_{BG+}$ treatment, 19 days post silencing (dps) were 39.2% of GS levels, which is comparable to other VIGS studies in wheat (Scofield et al., 2005; Van Eck et al., 2010). Scofield et al. (2005) reported that BSMV-mediated VIGS resulted in average *PDS* levels reduced to 31.5% of the control at 18 dps and average *RAR1* (a leaf rust resistance gene) expression reduced to 45.6% of the control at 12 dps. Van Eck et al. (2010) found the average expression of *WRKY53* silenced to 21.1% of control plants levels, 11 dps, by employing VIGS with a BSMV vector. Additionally, *(1,3;1,4)*-β-*glucanase* expression levels in GS+BSMV$_{BG+}$ were similar to the resistant treatment (GR). GS and GR show differential expression of *(1,3;1,4)*-β-*glucanase* upon RWA infestation, with a significant suppression occurring in the resistant NIL at 5 hpi (Figure 2.2) as well as 14 dpi (Figure 2.5).

This is in contrast to *(1,3)*-β-*glucanases* that have long been shown to be involved in resistant plant-pest/pathogen interactions, including responses to aphid feeding (Iglesias et al. 2000; Lapitan et al. 2007a; Smith and Boyko, 2007; Van der Westhuizen et al., 1998; Will and Van Bel, 2006). In fact, Lapitan et al. (2007a) reported increased (1,3)-β-

glucanase enzyme activity in resistant wheat genotype 93M370 (containing *Dn7*) compared to 'Gamtoos-S' under RWA-infested conditions.

Incompatible RWA reactions involve three phenotypic categories: antibiosis, antixenosis, and tolerance (Painter, 1958; Smith, 2005). To test whether the reduction of transcript levels in the susceptible background had a linear relationship with increased antibiotic effects, aphid reproduction was evaluated by calculating the number of aphid births per day for each foundress and the number of days from birth to the beginning of reproduction (pre-nymphipositional period, PNP). The silenced treatment showed a significant reduction in births per day compared to GS as well as an extended PNP, both of which exhibited the strongest linear relationships with *(1,3;1,4)*-β-*glucanase* transcript levels. This suggests that reduction of *(1,3;1,4)*-β-*glucanase* transcript abundance may affect the growth, development, and reproduction of the RWA, by inducing an antibiotic form of resistance (Painter, 1958).

Cell wall degrading enzymes, such as pectinase and cellulase, are present in the watery saliva of several aphid species, including greenbug (*Schizaphis graminum* Rondani), spotted alfalfa aphid (*Therioaphis maculata* Buckton), and green peach aphid (*Myzus persicae* Sulzer), and may function to facilitate aphid feeding and/or probing through enhanced stylet progress (Cherqui and Tjallingii, 2000; Giordanengo et al., 2010; Goggin, 2007; Harmel et al., 2008; Ma et al., 1990; Madhusudhan and Miles, 1993). Since aphids puncture and seal almost every cell their stylet encounters to provide probe-elicited directions en route to the phloem, reduction in plant cell wall degrading enzymes, such as (1,3;1,4)-β-glucanase, could impede probing and reduce feeding efficiency (Will

and Van Bel, 2006). Additionally, effector molecules can elicit the re-programming of host plant machinery to benefit the growth and propagation of the pest/pathogen (Giordanengo et al., 2010; Goggin, 2007). The increase in *(1,3;1,4)*-β-*glucanase* expression in RWA-infested susceptible wheat genotypes may be the result of aphid salivary elicitor manipulation of host gene expression, aiding in proliferation. Since the aphid per biomass ratios observed in the silenced treatment were approximately half of that in the GS treatment, it suggests that the silenced plants were not ideal food sources for the RWA, thereby indicating a component of antixenosis as well (Painter, 1958). However, it should be noted that under the non-caged conditions used in this study, whether the effect on aphid numbers per gram of dry plant weight was due to antixenosis or antibiosis was not determined and a whole-plant cage approach could help clarify this issue. The linear relationship between aphid reproduction and *(1,3;1,4)*-β-*glucanase* transcript levels suggests that susceptibility to RWA may be reduced by capitalizing on effects of *Dn7* through a downstream component, such as the suppression of *(1,3;1,4)*-β-*glucanase* expression.

The results of this study suggest that a decrease in *(1,3;1,4)*-β-*glucanase* transcript abundance under RWA infestation may lead to a less favorable environment for aphid growth and reproduction. We also investigated the effect of silencing on host symptom severity by assessing leaf rolling and chlorosis, as well as observing the degree of biomass inhibition. Based on our results, transcript expression influenced progression of and shared a direct linear relationship with chlorosis development, but had no impact on leaf rolling which suggests that the resistance conferred from expression reduction may

be independent from leaf rolling prevention. Chlorosis resulting from RWA feeding, has been shown to occur by way of chlorophyll catabolism that differs from that of natural senescence, but it is not as well understood (Ni et al., 2002). RWA feeding alters chlorophyll fluorescence and the efficiency of photosystem II, while decreasing chlorophyll *a*, *b*, chlorophyllide, and carotenoids in wounded areas of the plant (Burd et al., 2006; Ni et al., 2002; Wang et al., 2004). Compensation for the resulting pigment loss may come from non-wounded areas (Ni et al., 2002; Wang et al., 2004). Since there was reduced aphid reproduction and lower aphid per biomass ratios on GR and the silenced treatment, a reduction in the amount of aphid-damaged area could account for the decrease in chlorotic severity, but additional research will be needed to illuminate the specific role of *(1,3;1,4)*-β-*glucanase* in chlorosis development.

Highly infested plants exhibit significant reductions in dry weight; therefore, dry plant weight was measured in this study to obtain an estimate of plant tolerance (Burd and Burton, 1992; Mirik et al., 2009; Voothuluru et al., 2006). Tolerance involves survival under heavy levels of infestation and allows for the increased production of dry plant matter compared to that of susceptible genotypes (Painter, 1958; Voothuluru et al., 2006). This study showed an inverse relationship between *(1,3;1,4)*-β-*glucanase* transcript levels and total plant biomass. However, reduction of *(1,3;1,4)*-β-*glucanase* in GS to GR levels was insufficient for the silenced treatment to achieve the high weights observed in GR. This indicates that although *(1,3;1,4)*-β-*glucanase* expression may be involved in biomass accumulation, there must be other required factors. The silenced treatment showed greater above-ground and total biomass than susceptible treatments,

supporting previous findings that the role of wheat *(1,3;1,4)*-β-*glucanase* (with strong homology to barley E1) may be similar to rice *Gns1* and is stress-related, not developmentally regulated and required for growth (Lai et al., 1993; Nishizawa et al., 2003). It remains to be seen whether the increase in dry weight observed is directly related to biomass accumulation processes or an indirect result of reduced RWA numbers, allowing for allocation of energy towards plant growth and not nutrient manipulation by the aphid (Giordanengo et al., 2010).

The objectives of this study were to observe how BSMV-mediated VIGS of *(1,3;1,4)*-β-*glucanases* in a susceptible wheat genotype affected RWA reproduction and host symptom development. However, PTGS techniques such as VIGS can result in non-target silencing (Burch-Smith et al., 2004; Xu et al., 2006). PTGS involves species-specific degradation of endogenous mRNA, triggered by dsRNA (Baulcombe, 2004; Hannon, 2002; Plasterk, 2002). A DICER-like complex recognizes, binds to and cleaves dsRNA into 21- to 24-nucleotide small interfering RNAs (siRNA). These double stranded siRNA incorporate into an RNA-induced silencing complex (RISC) and guide the multi-subunit nuclease to endogenous mRNA sequences complimentary to the antisense strand of the siRNA. This leads to subsequent degradation of the target mRNA. The target mRNA also acts as a template for the RNA-dependent RNA polymerase (RdRp) for the creation of more dsRNA, resulting in amplification of silencing (Baulcombe, 2004; Hannon, 2002).

The issue of non-target silencing arises with mRNA sequence similarity between targets and non-targets. PTGS relies on nucleotide identity and siRNAs derived from

cleavage of the target sequence can result in suppression of non-target genes that share high identity with the siRNA (target) sequences (Scofield and Nelson, 2009; Xu et al., 2006). This can be beneficial or detrimental depending on the purpose of the investigation. In this study, we aimed to silence all endogenous *(1,3;1,4)*-β-*glucanases*. Without an annotated genome sequence, information on the number of *(1,3;1,4)*-β-*glucanases* in hexaploid wheat and the sequence variation present among them is limited (Scofield and Nelson, 2009). Based on the two available hexaploid wheat complete mRNA *(1,3;1,4)*-β-*glucanase* sequences, a VIGS construct was designed from a region highly conserved between them. As a consequence, the possibility of the non-target silencing of wheat *(1,3)*-β-*glucanases* increased. Results of the BLASTn query with the VIGS construct suggested that high sequence identity was likely limited to *(1,3;1,4)*- and *(1,3)*-β-*glucanases* (Table 2.2). The alignment of the VIGS construct with wheat *(1,3;1,4)*- and *(1,3)*-β-*glucanases* (Figure 2.3), revealed regions of identity that could potentially trigger silencing of *(1,3)*-β-*glucanase* as well . There were two major regions of the VIGS construct sequence, 56 bp and 47 bp in length, that shared between 78.6 and 87.2% identity with wheat *(1,3)*-β-*glucanases* (Figure 2.4). Within the smaller region was a 23 bp segment sharing perfect identity with the *(1,3)*-β-*glucanase* accessions (Table 2.3; Figure 2.4). A region of this size and similarity to *(1,3)*-β-*glucanases* could trigger non-target silencing (Burch-Smith et al., 2004; Fu et al., 2007; Xu et al., 2006).

Therefore, to increase the likelihood that all copies of *(1,3;1,4)*-β-*glucanase* were silenced, prevention of non-target silencing of *(1,3)*-β-*glucanases* was not avoided. The development of *(1,3)*-β-*glucanase*-specific RT-qPCR primers and amplification in the

cDNA synthesized in this study will help elucidate how *(1,3)*-β-*glucanase* transcript abundance was altered by the use of this VIGS construct. The relationship between *(1,3;1,4)*-β-*glucanase* and *(1,3)*-β-*glucanase* regarding host response to RWA is not known, so determining how *(1,3)*-β-*glucanase* was altered by silencing using this construct could help provide answers, if not more questions. If co-silencing of *(1,3)*-β-*glucanase* did occur, it presents an interesting research question. Since *(1,3)*-β-*glucanase* has been shown to be involved in resistance to RWA (Iglesias et al., 2000; Smith and Boyko, 2007; Van der Westhuizen et al., 1998; Will and Van Bel, 2006), why did the suppression of *(1,3;1,4)*-β-*glucanase* lead to increased RWA resistance if *(1,3)*-β-*glucanase* was also suppressed? If the most likely non-target *(1,3)*-β-*glucanase* was not silenced, *(1,3;1,4)*-β-*glucanase* may truly be a susceptibility factor that could be exploited as a potential avenue of aphid resistance. Since the presence of the *Dn7* resistance gene differentiates the GR and GS NILs, it follows that suppression of *(1,3;1,4)*-β-*glucanase* is one of the downstream effects of the resistance conferred by *Dn7*. Although the exact pathways and cellular responses induced by *Dn7* are not yet known (Botha et al., 2006), we suggest the putative involvement of a downstream component, *(1,3;1,4)*-β-*glucanase*, since suppression in the susceptible GS background to GR levels was associated with a more RWA-resistant phenotype. Subsequent investigation into the possible co-silencing of *(1,3)*-β-*glucanase* and a more detailed look into the role of *(1,3;1,4)*-β-*glucanase* in RWA susceptibility will provide insight into how *(1,3;1,4)*-β-*glucanase* suppression can be used in the implementation of novel RWA resistance in wheat breeding programs.

METHODS AND MATERIALS

Candidate gene selection

Microarray data published by Botha et al. (2010) was analyzed to identify genes up-regulated in GS compared to GR, 5 hours post-infestation (hpi) with RWA biotype 2. The data were sorted based on the logfold change in expression of GS/GR. The highest logfold change ratios correspond to transcripts with the greatest abundance in RWA-infested GS relative to RWA-infested GR. Candidate gene selection for this study was based on rank in the GS/GR logfold expression change list (Table 2.1). Identification of and sequence information for the candidate gene was obtained through searches of the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/) and HarvEST: Web (http://www.harvest-web.org/) databases. Verification of the differential expression of the candidate gene in GS and GR at 5 hpi was conducted using RT-qPCR (see *Real-time quantitative PCR* section for details)**.**

Silencing construct development and sequence analysis

VIGS primers were designed using Vector NTI v10 (Invitrogen Corp., Carlsbad, CA USA) to amplify a 338 bp fragment spanning a portion of the coding sequence into the 3' UTR of wheat *(1,3;1,4)*-β-*glucanase* cDNA sequences (accessions Z22874.1 and Z22873.1) (Table 2.4). Constructs were designed based on parameters described by Scofield et al. (2005) with the goal of silencing all endogenous copies of wheat *(1,3;1,4)*-β-*glucanase.* Somewhat similar basic local alignment search tool for nucleotide sequences (BLASTn) from NCBI (Zhang et al., 2000) and Geneious Pro™ v5.0.3 bioinformatics software (Drummond et al., 2010) were employed to investigate sequence

homology and pair-wise identity of the *(1,3;1,4)*-β-*glucanase* VIGS construct region to published sequences for determination of potential off-targets for silencing. The Geneious Pro™ v5.0.3 alignment was a global alignment with free end gaps and was constructed using the following parameters: cost matrix of 65% (5.0/-4.0), gap open penalty of 12, gap entension penalty of 3, and two refinement iterations.

The VIGS PCR product was digested with *Not*I (New England Biolabs, Ipswich, MA, USA) following transformation into a pGEM-T Easy Vector (Promega Corp., Madison, WI, USA) and ligated into the *Not*I digested BSMV γ genome plasmid, pSL038-1 (Cakir and Scofield, 2008). The cloned fragment was inserted immediately 3' to the γb gene in the γpSL038-1 plasmid (Cakir et al., 2010; Holzberg et al., 2002). Insert orientation was determined by PCR, employing plasmid-specific and insert-specific primer combinations to verify that the insert was in the antisense direction with respect to the γa and γb genes. Once orientation was determined, the cloned fragments were amplified from the plasmid and sequenced to verify the identity of the insert.

Plant materials and growth conditions

Near-isogenic lines of hexaploid wheat cultivar 'Gamtoos' were used for all experiments in this study. 'Gamtoos-R' (GR) is the RWA resistant line, containing the *Dn7* resistance gene, developed from the transfer of the 1BL.1RS wheat-rye (*Secale cereale* L.) translocation from accession 94M370 to the susceptible cultivar 'Gamtoos-S' (GS) (Anderson et al., 2003; Marais et al., 1994). BSMV inoculations were performed in the GS background with six plants per treatment. GS and GR plants, uninfected with BSMV, were used as the susceptible and resistant controls, also with six plants per

treatment. Each plant served as an independent biological replication since, at no point, were samples within a treatment pooled. Three seeds were planted in each six-inch pot to ensure germination of at least one plant per pot. On the day of BSMV inoculation, the extra plants were removed leaving one plant per pot. The remaining plants were selected so that they were all nearly the same age and developmental stage to reduce variability among and within treatments.

All plants were grown in the Colorado State University Insectary greenhouse on a 14/10 hr light cycle with approximately 24 °C days, with light intensities between 1100 – 1400 µM m^2s^{-1} and 20 °C nights. Since reports have suggested that *(1,3;1,4)*-β-*glucanase* expression may be affected by abiotic stresses including water-limited conditions (Konno et al., 2008; Roulin and Feller, 2001; Walti et al., 2002), care was taken to prevent water stress by placing pots in saucers and maintaining constant water levels. This served to reduced water-availability variation within and among treatments. This experiment was repeated, conducted spring 2009 and spring 2010, with similar trends observed. The results of the latter are reported herein.

BSMV inoculation and silencing

The α, β, γ RNA genome segments (both with and without the insert) were synthesized from cDNA using the mMessage mMachine T7 Kit (Ambion, Austin, TX, USA) followed by RNeasy Mini Kit spin column purification (Qiagen, Hilden, Germany). The tripartite BSMV genomes were combined in a 1:1:1 ratio (one µg each) and added to a FES buffer (0.1 M glycine, 1% w/v bentonite, 1% w/v celite, 0.06 M K_2HPO_4, 1% w/v tetrasodium pyrophosphate, 8.5 pH), used as the abrasive for viral

access into the plants. Two separate BSMV inoculations were prepared; one for silencing and the other, a viral control. The former, $BSMV_{BG+}$, consisted of α, β, and γ + *(1,3;1,4)*-β-*glucanase* insert, while the latter, $BSMV_{BG-}$, contained γ without any insert along with the α and β genomes. BSMV inoculation was conducted on 12-day old seedlings, at the 3-4 leaf stage, by rub-inoculation of the first and second leaves. A plastic covering was placed over the plants for 24 hours to provide a humid environment, suitable for viral penetration and replication, after which time the plastic was replaced by organza netting to prevent premature RWA infestation. Two of the six plants in the GS+$BSMV_{BG+}$ treatment were not silenced to levels significantly lower than that of GS or GS+$BSMV_{BG-}$ so they were removed from all but the regression analyses where they served as internal viral controls. Therefore, there were four biological replications for all treatment groups in each additional analysis.

Aphid infestation

Five days after BSMV inoculation, the third leaf of each plant was equipped with a custom clip aphid cage. Two apterous adult RWA biotype 2 aphids were placed in each cage. Upon the appearance of the first new nymph in each cage, the two original aphids were removed and the new nymph, hereafter referred to as the foundress, was observed for the duration of the experiment (Randolph et al., 2008). Following leaf caging, each plant was mass infested with ~150 RWA biotype 2 aphids that had been extracted from greenhouse host plants via a soil sieve (Collins et al., 2005b). Re-infestation occurred twice for all GS backgrounds and three times for the GR plants to maintain feeding pressure and encourage symptom development.

Data collection

Nymphs born to each foundress were counted and removed every 24 hours for 14 days to assess aphid reproduction (Randolph et al., 2008). To estimate antibiotic effects, the number of days to foundress reproduction or the pre-nymphipositional period (PNP), and the average number of aphid births per day per foundress were recorded (Lazzari et al., 2009; Webster, 1990). Upon conclusion of counting, above-ground vegetation was collected and placed in Berlese funnels to dry the plant matter and extract aphids for total plant aphid counts (Randolph et al., 2007). The dry above-ground plant matter was weighed for each plant. Roots were dried in an oven at ~43°C for 48 hours after being rinsed of excess particulate matter and weighed to determine dry root biomass. The above- and below-ground plant weights were used to determine effects of aphid feeding on each of the components separately and a total dry weight calculation was performed to determine the effect on overall plant growth as a modified tolerance assessment (Voothuluru et al., 2006). To assess a combination of antixenosis and antibiosis, the aphid/biomass ratio was calculated (Lazzari et al., 2009). Total plant aphids were kindly counted by Jack Mangels from the CSU Insectary.

On the day of tissue collection, the plant phenotypes were assessed via modified ratings of chlorosis and leaf rolling (Burd et al., 1993; Collins et al., 2005a; Webster et al., 1987). Chlorosis scores range from 1=healthy plants with small hypersensitive lesions to 9=dead or unrecoverable, while leaf rolling scores are on a scale of 1=completely flat leaves to 4=tightly rolled leaves with leaf trapping (Collins et al., 2005a).

Tissue collection and RNA extraction

Fourteen days after RWA infestation (19 days post-BSMV inoculation), the clip cages were removed and the area immediately surrounding and including the caged area, approximately 7.5 cm, was harvested and promptly frozen in liquid nitrogen. Tissues were stored at -80 °C prior to RNA extraction. Individual leaf samples were homogenized in liquid nitrogen and total RNA was extracted following the TRIzol reagent extraction protocol (Invitrogen, Carlsbad, CA, USA) with RNeasy Mini Kit purification, including on-column DNase I digestion post-extraction (Qiagen, Hilden, Germany).

Real-time quantitative PCR

RT-qPCR of RWA-infested GR and GS cDNA was conducted for verification of differential expression of *(1,3;1,4)-β-glucanase* at 5 hpi, based on results from the Botha et al. (2010) transcript profiling study. First-strand cDNA, previously created for verification of results from the same microarray study was kindly provided by Leon van Eck (CSU Crop Genomics Program, USA). The cDNA consisted of GR and GS samples from un-infested (0 hpi), 5, 24, and 48 hpi (with RWA biotype 2) tissues. However, since the results obtained from Botha et al. (2010) were from 5 hpi, only the uninfested (0 hpi) and 5 hpi samples were used for the verification of differential expression of *(1,3;1,4)-β-glucanase* in GR and GS. RT-qPCR was conducted using 2 ng of cDNA and PerfeCTa SYBR Green SuperMix for IQ (Quanta Biosciences, Gaithersburg, MD, USA) on a Bio-Rad MyiQ iCycler (Bio-Rad, Hercules, CA, USA). Each sample was amplified in triplicate to achieve three technical replications per sample. RT-qPCR products were

amplified with primers shown (Table 2.4) using the following iCycler protocol: 2 minute initial denaturation at 95 °C, 50 cycles of amplification involving 15 s denaturation 95 °C, preceding the 30 s annealing/extension step at 58 °C. Melt-curve analysis was performed on the RT-qPCR reactions to verify single product amplification by the *(1,3;1,4)-β-glucanase* RT-qPCR primers (Table 2.4). The samples were normalized to *UBQ5* and calibrated to the mean expression of uninfested GS (0 hpi) using relative quantification with efficiency correction (Pfaffl, 2001).

For determination of *(1,3;1,4)-β-glucanase* transcript abundance after BSMV-mediate VIGS, first-strand cDNA was synthesized using a blend of random hexamers and oligo (dTs) with the qScript cDNA synthesis kit (Quanta Biosciences, Gaithersburg, MD, USA) to promote synthesis of full-length transcripts. RT-qPCR was conducted using 2 ng of cDNA and PerfeCTa SYBR Green SuperMix for IQ (Quanta Biosciences, Gaithersburg, MD, USA) on a Bio-Rad MyiQ iCycler (Bio-Rad, Hercules, CA, USA). Each individual biological replication was amplified in triplicate to achieve three technical replications per biological replication. RT-qPCR products were amplified with the same RT-qPCR primers used for the Botha et al. (2010) microarray validation (Table 2.4) with the following iCycler protocol: 2 minute initial denaturation at 95 °C, 60 cycles of amplification involving 15 s denaturation 95 °C, preceding the 45 s annealing/extension step at 60 °C, for *(1,3;1,4)-β-glucanase*, or 57 °C for reference gene *18S*. Melt-curve analysis was performed on the RT-qPCR reactions to verify single product amplification by the *(1,3;1,4)-β-glucanase* RT-qPCR primers (Table 2.4). The transcript abundance of each individual biological sample was standardized across the

three corresponding technical replicates. Gene expression for all samples was normalized against constitutively expressed *18S* rRNA, and calibrated to the mean expression value of the samples in the GS treatment using relative quantification with efficiency correction (Pfaffl, 2001).

Statistical Analysis

Normality of the aphid reproduction and plant phenotype data was determined by proc UNIVARIATE using SAS v9.2 (SAS, 2008). Significance of the data and treatment comparisons were determined by one-way analysis of variance using SAS v9.2 proc GLM with the LSMEANS option, or proc NPAR1WAY combined with proc GLIMMIX, depending on the normality (SAS, 2008). Additionally, proc REG linear regression analyses were done to determine whether there was a linear relationship between the level of *(1,3;1,4)*-β-*glucanase* expression of the individual samples at 14 dpi and the RWA reproduction and plant phenotype data collected (SAS, 2008).

TABLES

Table 2.1 Top 15 candidate genes resulting from the logfold change in expression analysis of GS/GR, including the rank on the list, probe-set identifier, accession, and logfold expression change ratio. Analysis was performed on data from a previous transcript profiling study conducted by Botha et al. (2010).

Rank	Probe-set ID	Accession ID	Logfold expression change (GS/GR)
1	*Ta.10.2.S1_x_at	Z22873.1	2.7
2	Ta.14729.1.S1_at	CA681092	2.4
3	Ta.1120.1.S1_x_at	CA659877	2.3
4	Ta.20250.1.S1_at	CA676841	2.2
5	Ta.15173.1.A1_at	CA692265	2.0
6	TaAffx.53974.1.S1_at	CA687231	2.0
7	Ta.25334.1.A1_at	CD934949	1.9
8	*Ta.10.1.S1_a_at	Z22874.1	1.8
9	Ta.16407.1.S1_at	CK214726	1.8
10	TaAffx.525.1.S1_at	BQ802204	1.8
11	Ta.10.1.S1_x_at	Z22874.1	1.7
12	Ta.18241.1.S1_at	BG313234	1.7
13	TaAffx.105801.1.S1_s_at	BQ803283	1.6
14	TaAffx.28928.1.S1_at	CA654413	1.6
15	Ta.20516.1.S1_x_at	CA635174	1.4

*Chosen as candidate gene for this study.

Table 2.2 Results of the BLASTn search for sequences that resembled the VIGS construct used in this study, sorted by maximum identity. This query aligned sequence fragments of ≥ 11 bp that shared identity with the VIGS construct sequence.

Accession	Description	Query coverage	E-value	Max identity
AK332309.1	*T. aestivum* cDNA, clone Chinese Spring	100%	1.00E-171	100%
Z22873.1	*T. aestivum* b-glucanase mRNA, complete CDS	100%	1.00E-171	100%
Z22874.1	*T. aestivum* (1,3;1,4) b-glucanase mRNA, complete CDS	100%	2.00E-163	98%
DQ294235.1	*T. aestivum* (1,3;1,4) b-glucanase mRNA, partial cds	34%	8.00E-48	97%
AK251293.1	*H. vulgare* cDNA clone mRNA sequence	54%	1.00E-77	95%
M62740.1	*H. vulgare* 1,3-1,4-beta-D glucan 4-glucanohydrolase	54%	1.00E-77	95%
EF489499.1	*O. sativa* lichenase 2-precursor-like mRNA complete cds	55%	5.00E-76	94%
NM_001061912.1	*O. sativa* (Os05g0375400) mRNA, complete cds	55%	5.00E-76	94%
AC108874.2	*O. sativa* Japonica chromosome 5 complete sequence	55%	5.00E-76	94%
AK060867.1	*O. sativa* cDNA clone full insert sequence	55%	5.00E-76	94%
AF323610.1	*O. sativa* glucanase (GLU) mRNA, complete cds	55%	5.00E-76	94%
CT830265.1	*O. sativa* cDNA clone full insert sequence	55%	2.00E-74	94%
AY768944.1	*O. sativa* endo-1,3;1,4-beta-glucanase mRNA, complete	55%	2.00E-74	94%
AK121825.1	*O. sativa* cDNA clone full insert sequence	55%	2.00E-74	94%
M13237.1	*H. vulgare* b-glucanase mRNA	35%	2.00E-43	94%
DQ630522.1	*Bambusa oldhamii* b-endoglucanase mRNA, partial cds	15%	2.00E-12	94%
X58877.1	*O. sativa* (gns1) b-glucanase	55%	2.00E-73	93%
NM_001154989.1	*Z. mays* lichenase-2 mRNA complete cds	55%	7.00E-74	93%
AK252046.1	*H. vulgare* cDNA clone mRNA sequence	100%	2.00E-132	90%
X56260.1	*H. vulgare* (1,3;1,4)-beta-glucanase	100%	2.00E-132	90%
X52572.1	*H. vulgare* (1,3;1,4)-beta-glucanase	100%	2.00E-132	90%
X56775.1	*H. vulgare* (Glb 1) 1-3,1-4-beta-D-glucanase	98%	7.00E-131	90%
TAU30323	*T. aestivum* b-1,3-glucanase (Glc1) mRNA, complete cds	11%	1.00E-11	90%
XM_002440958.1	*Sorghum bicolor* hypothetical protein, mRNA	54%	9.00E-60	88%
NM_001156876.1	*Z. mays* lichenase-2 mRNA complete cds	54%	6.00E-56	87%
Z15131.1	*Avena sativa* b-glucanase mRNA	96%	2.00E-99	85%
CP000884.1	*Delftia acidovorans* SPH-1, complete genome	14%	0.11	80%
FP092155.1	*Phyllostachys edulis* cDNA clone full insert sequence	95%	1.00E-70	79%

Table 2.2 (continued) Results of the BLASTn search for sequences that resembled the VIGS construct used in this study, sorted by maximum identity. This query aligned sequence fragments of ≥ 11 bp that shared identity with the VIGS construct sequence.

Accession	Description	Query coverage	E-value	Max identity
XM_002459028.1	*Sorghum bicolor* hypothetical protein, mRNA	44%	1.00E-26	79%
AP003221.2	*O. sativa* Japonica Group genomic DNA, chromosome 1	44%	2.00E-23	79%
XM_002459029.1	*Sorghum bicolor* hypothetical protein, mRNA	44%	5.00E-25	78%
AY047606.1	*Sorghum bicolor* b-1,3-glucanase mRNA, partial cds	34%	6.00E-18	78%
AM181313.1	*S. cereale* glucan endo-1,3-b-D-glucosidase partial	33%	2.00E-16	78%
AM181309.1	*S. cereale* glucan endo-1,3-b-D-glucosidase mRNA	33%	2.00E-16	78%
EU024867.1	*Fragaria vesca* clone, complete sequence	24%	2.00E-05	77%
NM_001112264.1	*Z. mays* 1,3-b-glucanase protein mRNA	54%	6.00E-30	77%
AM181305.1	*S. cereale* mRNA for lichenase (glu-1 gene)	53%	1.00E-27	76%
EU725049.1	*Z. mays* b-1,3 glucanase (pr6) gene, complete cds	54%	8.00E-29	76%
XM_002459031.1	*Sorghum bicolor* hypothetical protein, mRNA	54%	1.00E-27	75%
AF055328.1	*H. vulgare* glucan endo-1,3-b-glucosidase (I), complete	31%	2.00E-10	75%
M96938.1	*H. vulgare* glucan endo-1,3-b-glucosidase (I), mRNA	31%	2.00E-10	75%
NM_001051898.1	*O. sativa* (Os01g0942300) mRNA, partial	53%	7.00E-23	74%
AK063126.1	*O. sativa* Japonica Group cDNA clone full insert	53%	7.00E-23	74%
AK249551.1	*H. vulgare* cDNA clone mRNA sequence	50%	4.00E-20	74%
M96939.1	*H. vulgare* glucan endo-1,3-b-glucosidase (V) mRNA	50%	4.00E-20	74%
AK334061.1	*T. aestivum* cDNA, clone Chinese Spring	31%	1.00E-08	74%
XM_002441352.1	*Sorghum bicolor* hypothetical protein, mRNA	52%	2.00E-16	73%
AF112967.1	*T. aestivum* b-1,3-glucanase precursor (Glb3) mRNA	53%	2.00E-18	72%
AK249935.1	*H. vulgare* cDNA clone mRNA sequence	53%	6.00E-18	72%
FP099037.1	*Phyllostachys edulis* cDNA clone full insert sequence	53%	8.00E-16	72%
DQ431670.1	*Lilium hybrid* cv. 'Star Gazer' b-1,3-glucanase mRNA	35%	1.00E-06	72%
AY612193.1	*H. vulgare* b-1,3-glucanase mRNA, complete cds	53%	2.00E-17	71%
XM_002459036.1	*Sorghum bicolor* hypothetical protein, mRNA	55%	8.00E-16	71%
FP093777.1	*Phyllostachys edulis* cDNA clone full insert sequence	53%	1.00E-14	71%
AK248896.1	*H. vulgare* cDNA clone mRNA sequence	46%	4.00E-07	69%
AJ890250.1	*T. aestivum* glucan endo-1,3-b-D-glucosidase partial	51%	6.00E-14	68%

Table 2.3 The number of identical sites and pair-wise identities between the VIGS construct and wheat *(1,3)*-β-*glucanase* sequences in two major regions of similarity. Segment A represents the 56 bp segment spanning bases 1-56 of the VIGS construct, while segment B (47 bp) includes bases 75-122 within the construct.

Segment	Accession	Description	Identical sites	Pairwise identity (%)
A	DQ090946.1	b-1,3-glucanase mRNA	44/56	78.6
	DQ078255.1	b-1,3-glucanase mRNA	44/56	78.6
B [t]	DQ090946.1	b-1,3-glucanase mRNA	41/47	87.2
	DQ078255.1	b-1,3-glucanase mRNA	41/47	87.2

[t] Within this region is a 23 bp segment with 100% pair-wise identity compared to the VIGS construct.

Table 2.4 Wheat *(1,3;1,4)*-β-*glucanase* primers used for this study.

Accession	Purpose	Length (bp)	Primer sequence
Z22873.1/Z22874.1	RT-qPCR	156	5'-GCCGCTCATGGCCAACATCTAC-3'
			5'-TAGAAGGCGTCCACGGTGGTGT-3'
	VIGS	338	5'-CCAACGCCAGGATCTACAAC-3'
			5'-TACGCATCTGAGCTGCCTCA-3'

Table 2.5 Aphid reproduction and plant phenotype data for each treatment (reported with standard errors). Means within a column annotated with the same letter were not significantly different at the 0.05 probability level.

Treatment	Average births day^{-1}	Chlorosis	Pre-nymphipositional period (PNP)	Leaf rolling
GS+BSMV$_{BG+}$	0.92 ± 0.10^{a}	2.50 ± 0.29^{a}	9.00 ± 0.00^{a}	4.0 ± 0.00^{a}
GS+BSMV$_{BG-}$	1.46 ± 0.11^{b}	6.75 ± 0.95^{b}	7.75 ± 0.48^{b}	4.0 ± 0.00^{a}
'Gamtoos-S'	1.67 ± 0.07^{b}	7.00 ± 0.41^{b}	7.25 ± 0.48^{b}	4.0 ± 0.00^{a}
'Gamtoos-R'	0.65 ± 0.04^{c}	2.00 ± 0.00^{a}	10.00 ± 0.00^{a}	2.0 ± 0.00^{b}

Table 2.6 Total plant and individual component dry weights for the treatments (reported with standard errors). Means within a column followed with the same letter were not significantly different at the 0.05 probability level.

Treatment	Root weight (g)	Above-ground weight (g)	Total dry weight (g)
GS+BSMV$_{BG+}$	0.165 ± 0.04[a]	0.480 ± 0.09[a]	0.645 ± 0.13[a]
GS+BSMV$_{BG-}$	0.085 ± 0.02[a]	0.213 ± 0.03[b]	0.297 ± 0.05[b]
'Gamtoos-S'	0.085 ± 0.01[a]	0.213 ± 0.03[b]	0.297 ± 0.04[b]
'Gamtoos-R'	0.275 ± 0.04[b]	0.918 ± 0.13[c]	1.193 ± 0.17[c]

FIGURES

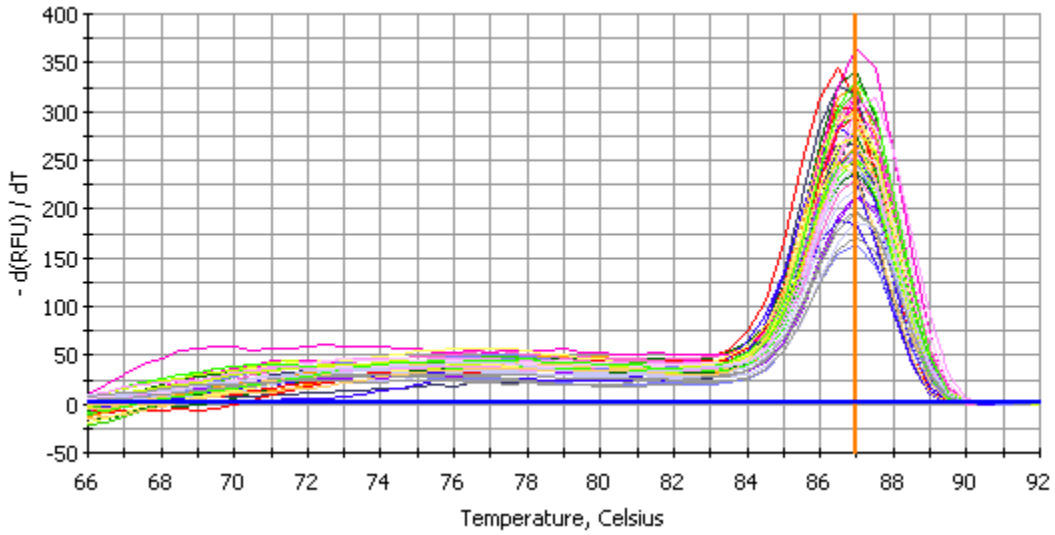

Figure 2.1 The melt curve from the RT-qPCR reactions of the target gene (*(1,3;1,4)-β-glucanase*) performed to validate the differential expression in GR and GS reported in the transcript profiling study conducted by Botha et al. (2010). This curve verifies that the *(1,3;1,4)-β-glucanase* RT-qPCR primers designed for use in our study amplify only *(1,3;1,4)-β-glucanase* in GR and GS cDNA leaf samples. GR and GS cDNA from non-infested, 5, 24, and 48 hours post-RWA biotype 2 infested tissues were subjected to RT-qPCR. The samples were amplified in triplicate.

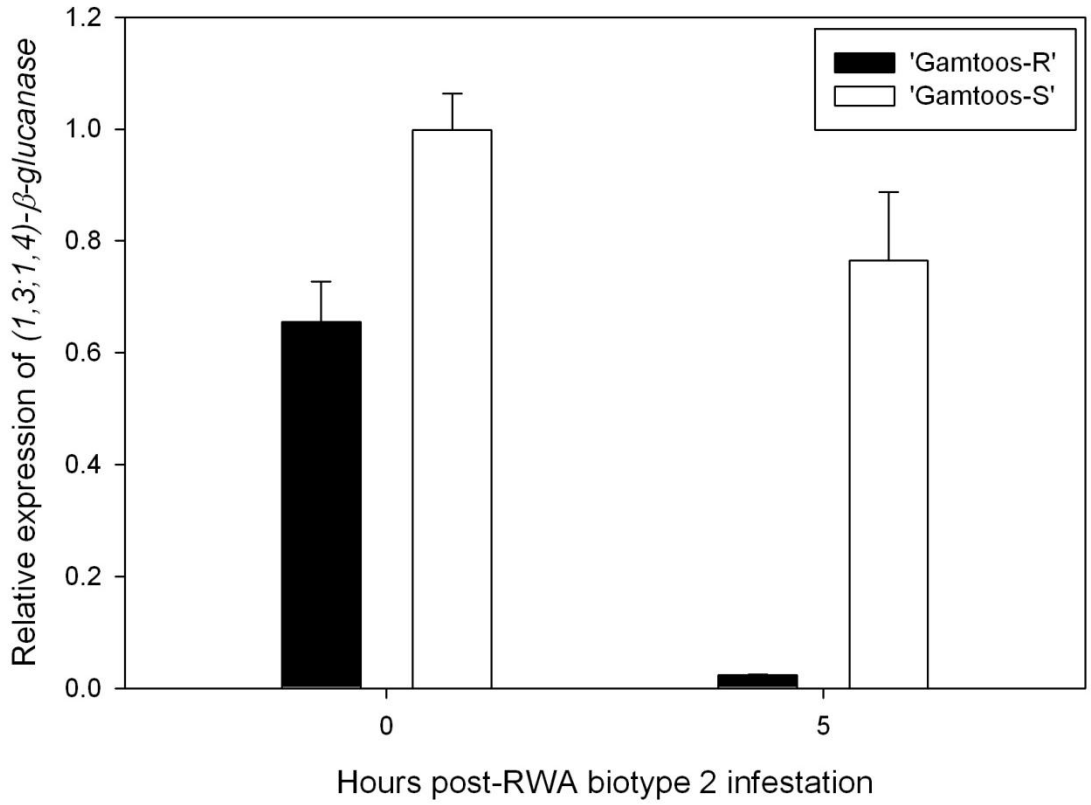

Figure 2.2 *(1,3;1,4)-β-glucanase* transcript expression in GR and GS tissues collected at 0 and 5 hours post-infestation (hpi) with RWA biotype 2. The differential expression between GR and GS at 5 hpi verifies the results from the previous transcript profiling study conducted by Botha et al. (2010). GR and GS cDNA from non-infested, 5, 24, and 48 hpi tissues were subjected to RT-qPCR. Since the data obtained from Botha et al. (2010) was for 5 hpi, only the uninfested (0 hpi) and 5 hpi samples were used for the differential expression verification. RT-qPCR values for each sample was averaged across three technical replicates, calibrated against uninfested GS (0 hpi) and normalized with *UBQ5* with relative quantification and efficiency correction.

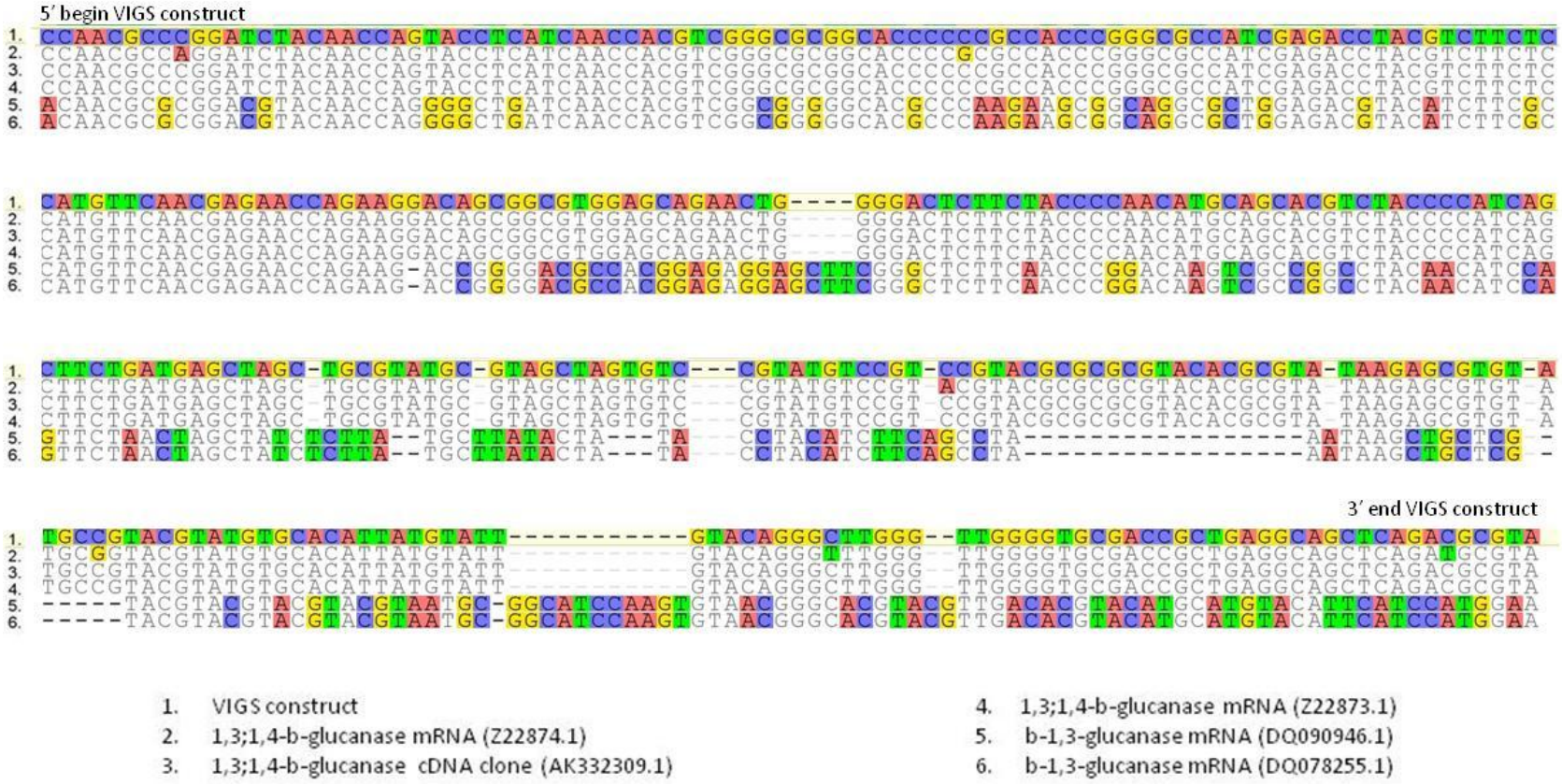

Figure 2.3 Details of the wheat *(1,3)-* and *(1,3;1,4)-β-glucanase* alignment of the 338 bp VIGS construct region created with Geneious v5.0.3, using the VIGS construct as the reference sequence. Color blocks for sequences 2-6 indicate where the sequence differs from the VIGS construct sequence.

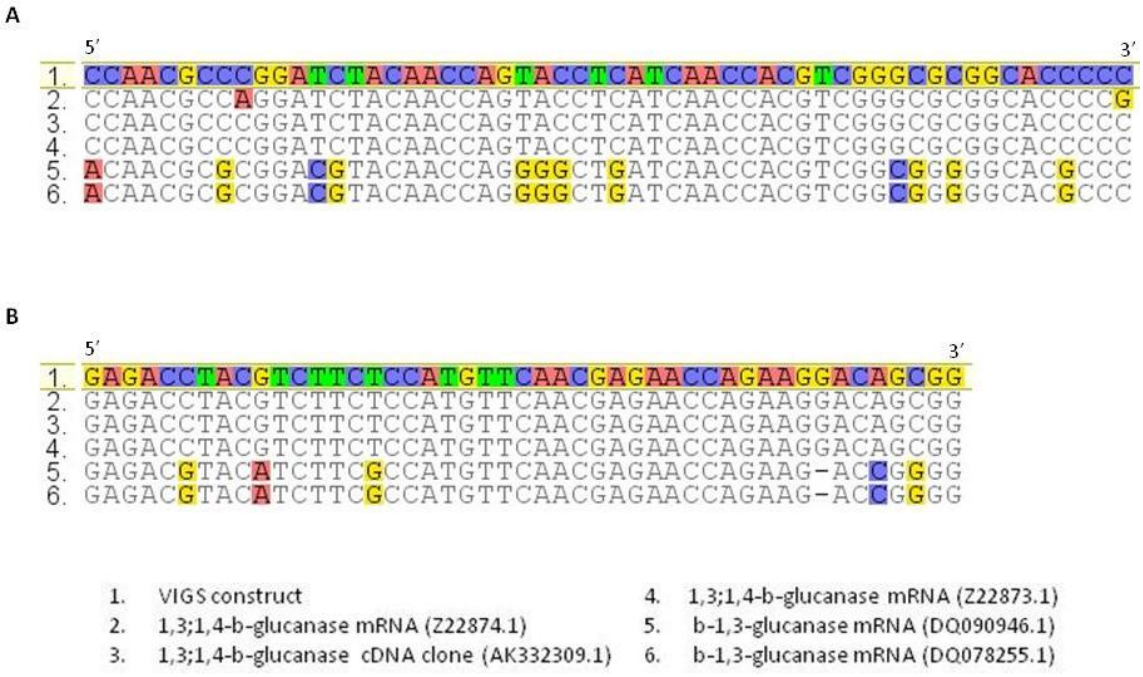

Figure 2.4 Two segments within the VIGS construct-wheat glucanase alignment consisting of the largest regions of shared identity; **A,** a 56 bp segment at the 5' end of the VIGS construct (bases 1-56) and **B,** a 47 bp segment corresponding to bases 75-122 of the VIGS construct. This is an extraction from the alignment created using Geneious v5.0.3 and color blocks present in sequences 2-6 represent base pair differences from the VIGS construct reference sequence.

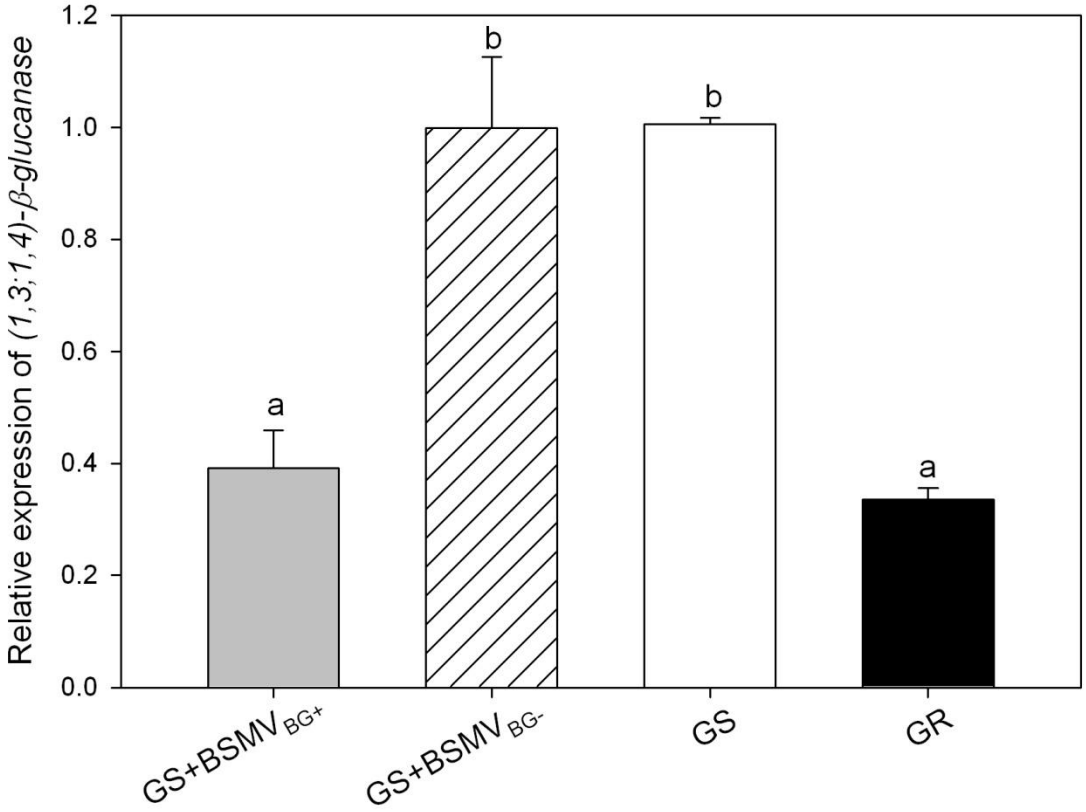

Figure 2.5 BSMV-mediated VIGS silencing efficiency as revealed by differences in *(1,3;1,4)-β-glucanase* transcript abundance, relative to the susceptible GS treatment. RT-qPCR values were standardized across the four biological replications, amplified in triplicate, within each treatment. All values were calibrated against the mean expression value of the susceptible GS treatment and normalized to *18S* rRNA. Means with the same letter were not significantly different at the 0.05 probability level.

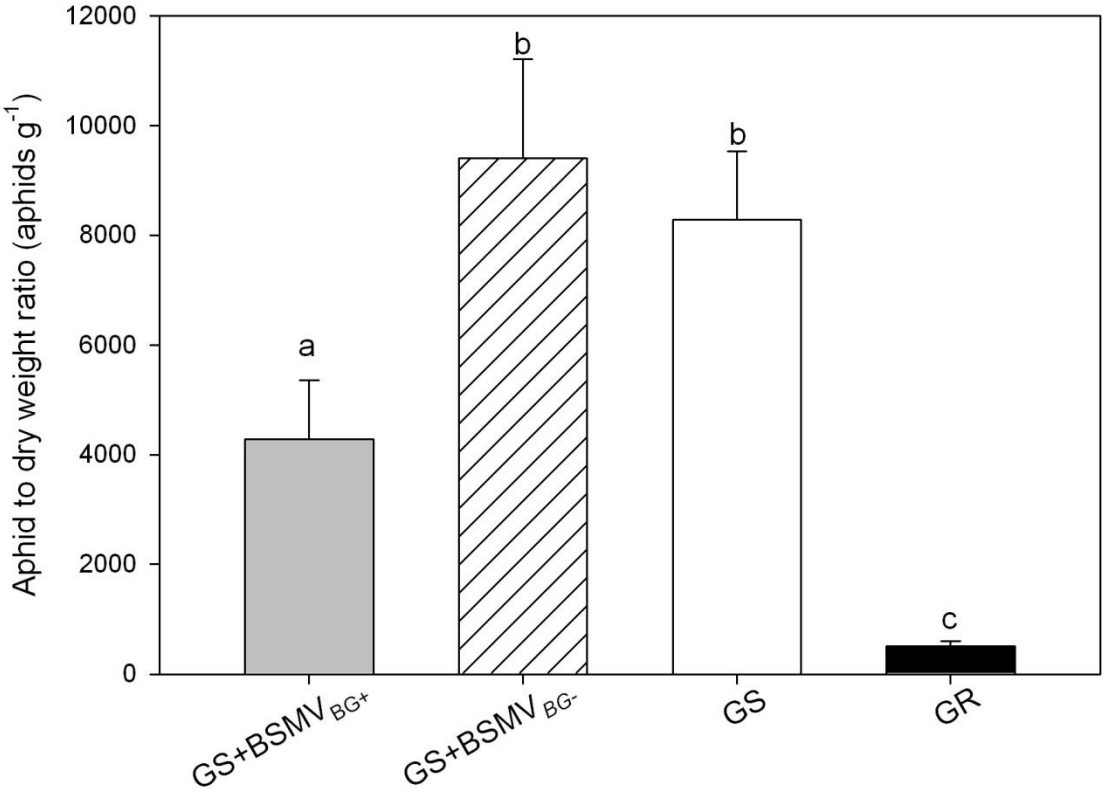

Figure 2.6 Number of aphids per gram of dry plant weight, averaged across four biological replications per treatment. Means with the same letter were not significantly different at the 0.05 probability level.

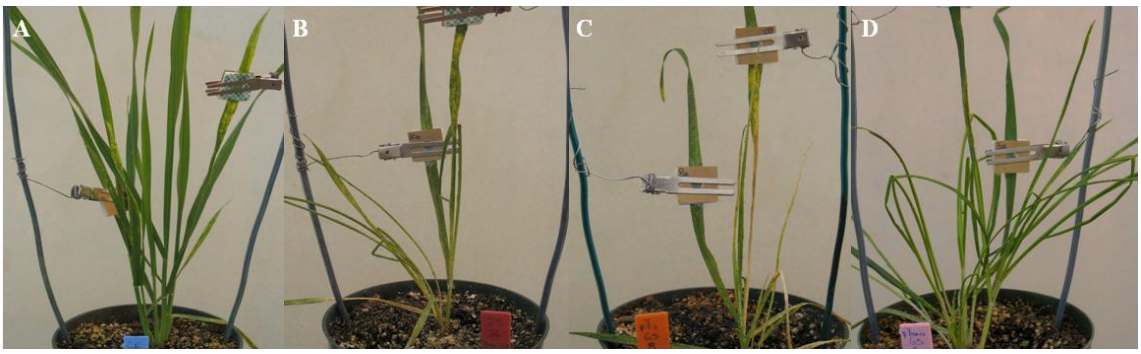

Figure 2.7 Phenotypes of one representative plant per treatment 19 days post-BSMV inoculation (14 days post-RWA infestation); **A** 'Gamtoos-R' (GR), **B** 'Gamtoos-S' (GS), **C** GS+BSMV$_{BG-}$ and **D** GS+BSMV$_{BG+}$.

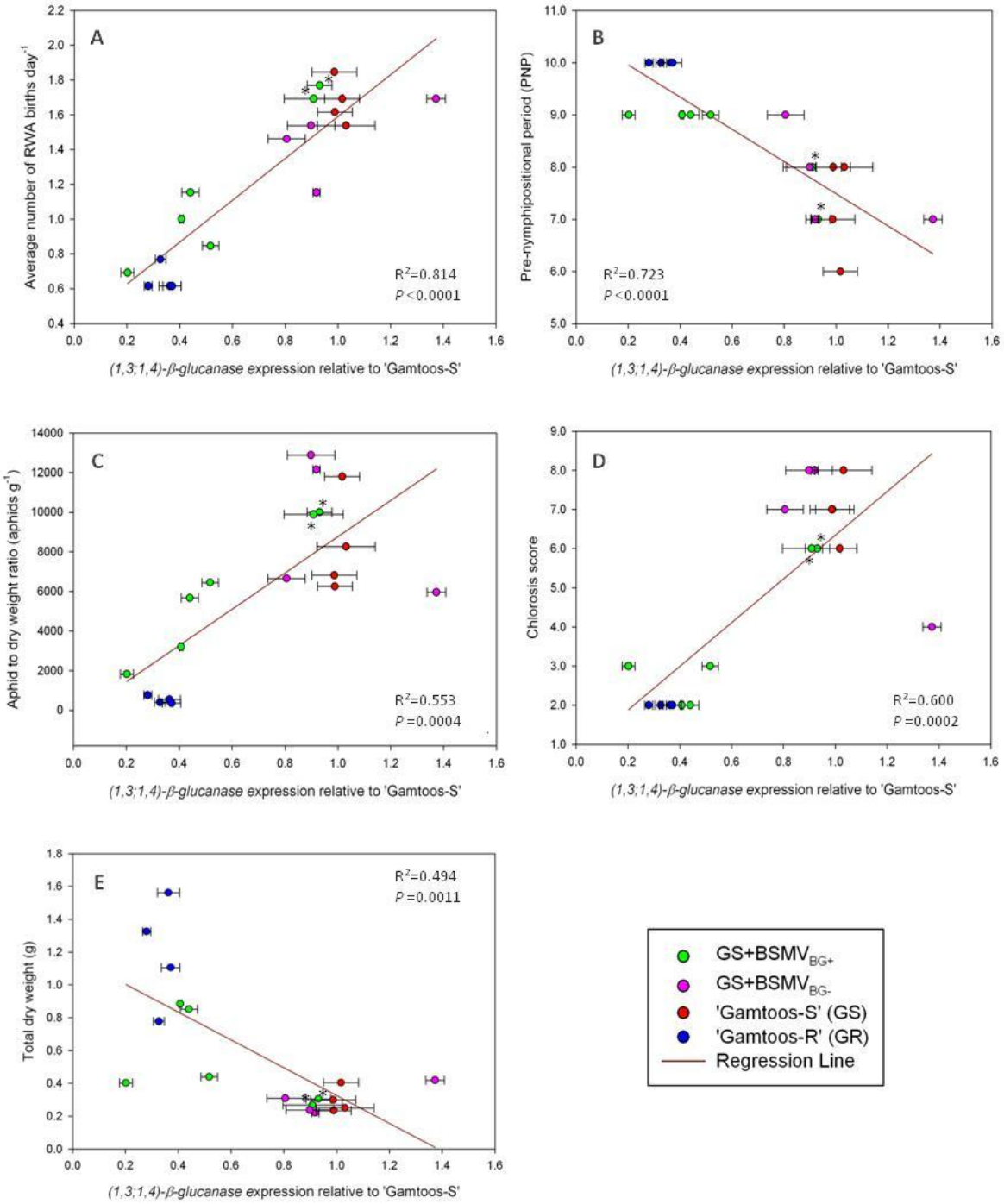

Figure 2.8 Linear relationships observed between *(1,3;1,4)*-β-*glucanase* transcript expression at 14 dpi and; **A** average number of births per day, **B** pre-nymphipositional period (PNP), **C** total number of aphids per gram of dry plant weight, **D** chlorosis severity, and **E** total dry plant weight. *Indicates plants in the GS+BSMV$_{BG+}$ treatment that were not silenced at time of tissue collection, included as internal viral controls.

CHAPTER THREE

INHERITANCE AND GENETIC MAPPING OF RUSSIAN WHEAT APHID RESISTANCE IN IRANIAN LANDRACE ACCESSION PI 626580

ABSTRACT

Russian wheat aphid (RWA), *Diuraphis noxia* (Kurdjumov), is a significant insect pest of wheat (*Triticum aestivum* L.) and has had a major economic impact worldwide, especially on winter wheat in the western part of the United States. Production of resistant cultivars remains the most viable method for RWA control, but with the continuing emergence of new RWA biotypes virulent to existing resistance genes, there is need for the discovery of new sources of resistance. Iranian landrace accession PI 626580 has shown high levels of resistance to RWA biotype 1 and biotype 2, yet the inheritance and chromosomal location of this resistance is unknown. The objectives of this study were to determine the inheritance of resistance, identify closely linked markers, and map the chromosomal location of potentially novel RWA resistance found in Iranian landrace accession PI 626580. Additionally, due to the high trichome density of this

plant introduction, we set out to determine whether this trait contributed to the resistance conferred by PI 626580. Trichome density was not involved in the RWA resistance of this plant introduction, since trichome densities of the homozygous resistant, heterozygous, and homozygous susceptible phenotypic classes were not significantly different (P=0.795). Bulked segregant analysis with a mapping population of 154 F_2 individuals, developed from a single plant selection made from PI 626580 and 'Yuma' (a susceptible wheat cultivar), was employed. RWA resistance in PI 626580 was conferred by a single dominant gene provisionally designated as *Dn10*. Linkage mapping analysis identified three SSR markers, *Xbarc214*, *Xgwm473* and *Xgwm437*, proximally linked to *Dn10* near the centromere on the short arm of chromosome 7D, at distances of 12.9 cM, 16.0 cM, and 19.2 cM, respectively. The marker order, with respect to resistance in PI 626580, was verified by a quantitative single factor analysis of variance which revealed significant marker associations with chlorosis and leaf rolling under greenhouse infestation (P<0.0001 for all three markers). *Xbarc214* was the highest contributor to phenotypic variance for both the chlorosis and leaf rolling QTL (R^2=64.6 and 64.8%, respectively). *Xgwm437* contributed 60.8% of chlorosis variation and 60.7% of the variation observed in leaf rolling. *Xgwm473* was shown to be between *Xbarc214* and *Xgwm437*, contributing 63.1% of the symptom variance for chlorosis and 64.2% for leaf rolling. *Dn10*, a new resistance gene found in PI 626580 could be used alone or by pyramiding with other *Dn*-genes to develop cultivars with improved RWA resistance.

INTRODUCTION

The Russian wheat aphid (RWA), *Diuraphis noxia* (Kurdjumov), is an important insect pest of wheat (*Triticum aestivum* L.) and barley (*Hordeum vulgare* L.) and has had a major economic impact worldwide, especially on winter wheat in the western part of the United States (Burd et al., 2006; Haley et al., 2004; Weiland et al., 2008). Since 1987, over $1 billion in economic losses due to the RWA have accrued with 60% of that reported in southeastern Colorado, western Kansas, southwestern Nebraska, and the panhandles of Texas and Oklahoma (Smith et al., 2004). Phenotypic damage to the host plant includes chlorosis and longitudinal streaking, leaf rolling, head trapping, and stunted growth (Botha et al., 2006; Burd et al., 1998; Franzen et al., 2008; Smith et al., 1992). When RWA are abundant, they can greatly damage wheat and barley in a very short period of time (Mirik et al., 2009), resulting in 100% reductions in yield and/or death (Elliot et al., 2007). A single biotype existed in the U.S. until a second, more virulent biotype (RWA biotype 2) was discovered in Colorado in 2003 and was recognized to be virulent to most RWA resistant sources at the time (Haley et al., 2004; Porter et al., 2005). Since the discovery of RWA biotype 2, six more biotypes have been identified although biotype 2 remains the most virulent, the most common type found in Colorado, and the largest RWA threat to wheat and barley growers in the western U.S. (Randolph et al., 2008).

Methods for RWA control have included insecticide application (requiring complete coverage), bio-control agents (such as the aphid parasitoid wasp, *Diaeretiella rapae*), and the production and deployment of resistant cultivars (Baker et al., 2003; Burd et al., 2006;

Hodgson and Karren, 2008; Morrison and Peairs, 1998). The economic and environmental cost of insecticide use and the possibility of the emergence of insecticide-resistant aphids are potential deterrents to chemical control of RWA (Burd et al., 2006). Bio-control has its own complications. RWA reproduction out-paces that of most of its bio-control species and since bio-control agent populations tend to expand more slowly, by the time they reach effective sizes RWA has already imparted significant damage to their hosts (Adisu and Freier, 2003; Tagu et al., 2008). Additionally, aphid-induced leaf rolling physically protects RWA from contact pesticides and/or bio-control agents (Haile et al., 1999). Non-native introductions are at high risk for becoming invasive species that, due to difficulties in eradication, present a whole new set of challenges (Andreu and Vila, 2010). Therefore, production of resistant cultivars remains the most viable option for control of RWA. With the continuing emergence of new biotypes virulent to existing resistance genes, however, there is need for the identification of new sources of resistance and implementation of more durable resistance via broad-spectrum resistance or pyramiding known resistance genes (Liu et al., 2002; Melchinger, 1990).

Aphid resistance can be conferred polygenetically or monogenetically and the genes are often located in resistance gene clusters within specific chromosomal regions (Dogimont et al., 2010). To date, 12 named RWA resistance genes (*Dn*-genes) have been identified; *Dn1* and *Dn2* (Du Toit, 1987, 1988, 1989), *dn3* and *Dn4* (Nkongolo et al., 1991a, 1991b), *Dn5* (Marais and Dutoit, 1993), *Dn6* (Saidi and Quick, 1996), *Dn7* (Marais et al., 1994), *Dn8*, *Dn9*, and *Dnx* (Liu et al., 2001), *Dny* (Smith et al., 2004), and resistance conferred by the cereal introduction, CI 2401 (consisting of two *R*-genes; one

allelic to *Dn4* and the other different from known *Dn* genes) (Collins et al., 2005a; Dong et al., 1997; Voothuluru et al., 2006).

Chromosomal locations of many of these genes have been identified, for example *Dn1*, *Dn2*, *Dn5*, *Dn6*, *Dn8*, *Dnx*, and the unique CI 2401 gene have been mapped to wheat chromosome 7D (Du Toit et al., 1995; Heyns et al., 2006; Liu et al., 2001, 2002, 2005; N. Lapitan, CSU Crop Genomics Program, USA, unpublished). Both *Dn4* and *Dn9* are located on chromosome 1D, with the former on the short arm and the latter on the long arm (Arzani et al., 2004; Liu et al., 2001, 2002; Ma et al., 1998). *Dn7* is located on the 1BL.1RS wheat-rye translocation (Marais et al., 1994). There have been many attempts to determine the allelic relationship between the *Dn*-genes located on 7D, but the results have been inconsistent (Miller et al., 2001). The most recent study conducted by Liu et al. (2005) helped resolve confusion from previous conflicting reports (Du Toit, 1989; Marais and Dutoit, 1993; Saidi and Quick, 1996) by concluding that *Dn1*, *Dn2*, *Dn5*, *Dn6*, and *Dnx* were either allelic at the same locus or located in a *Dn*-gene cluster, tightly linked to each other and to SSR marker *Xgwm111*. Despite the identification and characterization of these genes, all except *Dn7* are ineffective against the most virulent biotype, RWA biotype 2, which reinforces the need for the identification of new sources of resistance (Collins et al., 2005a; Haley et al., 2004; Peng et al., 2007; Smith et al., 2004).

PI 626580 is a highly pubescent, Iranian wheat landrace accession found to be resistant to both RWA biotypes 1 and 2 and thought to confer novel resistance (S. Haley, CSU Wheat Breeding Project, USA, unpublished). Haley (unpublished) screened over

7,000 Iranian wheat landrace accessions for resistance to RWA biotype 1 and biotype 2 and identified 10 for further study based on geographic origin within Iran. Each of the 10 accessions selected were crossed to each other and the segregation ratios of the F_2 progeny were assessed for evidence of gene independence (fitting a 15:1 ratio), epistasis (fitting a 13:3 ratio since phenotypes were skewed towards resistance) or allelism (lack of segregation of resistance). Of the nine crosses involving PI 626580, allelism (either conference of resistance via the same gene, allelic forms of the same gene, or tightly linked genes) was only indicated twice suggesting the resistance in PI 626580 may be unique among the examined Iranian accessions.

Genetic linkage mapping of pest/pathogen resistance and agronomic traits plays a large role in the advancement of cultivar development through the use of marker-assisted selection (MAS) (Collard and Mackill, 2008; Xu and Crouch, 2008). MAS permits the genotypic screening of resistance under conditions when it is impossible, undesirable, prohibitively expensive, or inefficient to perform phenotypic screens (Collard and Mackill, 2008; Xu and Crouch, 2008). MAS also allows for the pyramiding of genes, which in the case of biotic resistance can create more durable resistance and/or resistance to several different pests or pathogens (Liu et al., 2002; Melchinger, 1990). For effective use in MAS, DNA markers should meet several requirements. These include reliability with tight linkage and/or flanking the gene of interest, the ability to use low quantity and quality of DNA, inexpensive implementation, high levels of polymorphism, and detection with high-throughput capacity (Collard and Mackill, 2008; Paux et al., 2010).

Many different types of markers have been employed in the mapping of monogenic RWA resistance genes, including restriction fragment length polymorphic (RFLP) markers (Ma et al., 1998) and polymerase chain reaction (PCR)-based markers including random amplified polymorphic DNA (RAPD) (Myburg et al., 1998) and microsatellite or simple sequence repeat (SSR) markers (Anderson et al., 2003; Arzani et al., 2004; Lapitan et al., 2007b; Liu et al., 2001, 2002, 2005; Miller et al., 2001; N. Lapitan, CSU Crop Genomics Program, USA, unpublished; Peng et al., 2007). SSRs exhibit higher levels of polymorphism than most other markers and are often co-dominant (Guyomarc'h et al., 2002; Huang et al., 2002b; Röder et al., 1998). The high-throughput potential of SSRs is enhanced by capillary electrophoresis platform development and that their implementation requires only small amounts of DNA (Guyomarc'h et al., 2002; Somers et al., 2004). With their ease of use, reliability and effectiveness in species with limited genetic variation, SSRs are the currently the most widely utilized marker for RWA (and other pests) resistance genetic mapping studies (Liu et al., 2002; Nicot et al., 2004).

Insertion site-based polymorphic (ISBP) markers are created from bacterial artificial chromosome (BAC)-end sequences by designing primer pairs where one primer flanks a transposable element (TE) and the other is nested within the TE (Devos et al., 2005; Paux et al., 2010; Wanjugi et al., 2009). Of the ~80-90% repetitive DNA that comprises the hexaploid wheat genome, ~60-70% of that are TEs (Devos et al., 2005; Wanjugi et al., 2009). They are found in eu- and heterochromatin in high copy number and show high levels of within and among species variation in their insertion-sites (Paux et al., 2006). Since ISBPs are also PCR-based markers, they lend themselves to high-throughput

genotyping as well (Paux et al., 2010; Somers et al., 2004). Currently ISBPs are available for chromosome 5B and 7DS, the latter near the centromere (H. Simkova, Institute of Experimental Botany, Czech Republic, unpublished; Paux et al., 2008). ISBPs are beginning to be employed in genetic linkage map studies involving a variety of different crops such as rice, barley, and wheat (Paux et al., 2008, 2010; Wanjugi et al., 2009).

The objectives of this study were to determine the inheritance of resistance, identify closely linked markers, and map the chromosomal location of potentially novel RWA resistance found in Iranian landrace accession PI 626580. Additionally, due to the high trichome density of this plant introduction, we set out to determine whether this trait contributed to the resistance conferred by PI 626580.

METHODS AND MATERIALS

Plant Materials

PI 626580 was collected from Yazd, Iran (Figure 3.1) in October of 1997 and was donated by Dr. Calvin O. Qualset (University of California-Davis) to the USDA-ARS National Small Grains Collection (NSGC) (USDA-ARS, 2010). Seed of PI 626580 for this study was kindly provided by Harold E. Bockelman, curator of the NSGC. The mapping population was developed from a single plant selection made from PI 626580 (designated PI 626580-4) as the female parent and 'Yuma' (PI 559720), a wheat cultivar susceptible to all known RWA biotypes, as the male parent. F_1 hybrids were selfed, producing F_2 progeny. The 154-F_2 plants were grown under ambient greenhouse conditions and seed from individual plants was harvested to produce $F_{2:3}$ families. Phenotypic RWA resistance evaluations were performed on the $F_{2:3}$ families.

Trichome densities for each of the 154-F_2 individuals were determined for a between-class comparison by calculating the number of trichomes present on a single mm^2 section of leaf tissue. This service was kindly provided by Tyler Keck at the Colorado State University Insectary. Normality of the trichome data was assessed using SAS v9.2 proc UNIVARIATE (SAS, 2008). The significance of trichome density segregation by phenotypic class (homozygous resistant, homozygous susceptible, or heterozygote) was determined by a one-way analysis of variance (ANOVA) using SAS v9.2 proc GLM with the LSMEANS option (SAS, 2008).

RWA Screening

RWA screening was done in the Colorado State University Insectary greenhouse under ambient conditions (14 hr, ~25.5 °C days with light intensities between 1100-1400 µM m^2 s^{-1} and 10 hr, ~20 °C nights). Twelve to fifteen seeds per $F_{2:3}$ family were planted, one row per family, in 52 cm x 25.5 cm flats. There were 14 flats per replication and a total of two replications; the second with the entries randomized differently from the first replication. Seven-day old seedlings were infested in the spring of 2009 with RWA biotype 2 by scattering aphids evenly across the flats. PI 626580-4 and 'Gamtoos-R' (containing the *Dn7* resistance gene and resistant to RWA biotypes 1 and 2) were included as resistant controls, while 'Yuma' served as the susceptible control. Plants were clipped to the height of 10-12 cm to better distinguish individuals and observe leaf rolling.

Symptom development was assessed 26 days post-infestation (dpi) via modified ratings of chlorosis and leaf rolling (Burd et al., 1993; Collins et al., 2005a; Webster et al., 1987). Chlorosis scores range from 1=healthy plants with small hypersensitive lesions to 9=dead or unrecoverable, while leaf rolling scores are on a scale of 1=completely flat leaves to 4=tightly rolled leaves with leaf trapping (Collins et al., 2005a). Plants with chlorosis scores of ≤ 4 and leaf rolling scores ≤ 2 were considered resistant and those exhibiting chlorosis ≥ 5 and leaf rolling ≥ 3 were considered susceptible by allowing minimal leaf rolling for the resistant classification. The F_2 individuals were assigned a phenotypic class (homozygous resistant, heterozygous, or homozygous susceptible) based on the number of resistant and susceptible individuals observed in the $F_{2:3}$ families, under

RWA infestation. Homozygous resistant and susceptible designations were only given to F_2 individuals when all (or all but one) of the plants in the $F_{2:3}$ family were given the corresponding designation.

DNA Extraction and Marker Analysis

Genomic DNA was extracted from leaves of the 154-F_2 individuals and the parents. Approximately 7.5 cm of tissue from the newest leaf was collected in 96-well plates, frozen in liquid nitrogen, and stored at -80 °C. DNA was extracted as previously described (Pallotta et al., 2003), modified by Somers and Chao (2006). Resistant and susceptible bulks were produced by pooling, in equal concentrations, DNA from 7-F_2 plants categorized as homozygous resistant and 10-F_2 plants categorized as homozygous susceptible, based on $F_{2:3}$ family evaluations. Bulked segregant analysis was employed to identify molecular markers that were polymorphic between the parents and between the resistant and susceptible bulks (Michelmore et al., 1991). Markers identified as polymorphic were then screened across all 154-F_2 individuals to determine the genetic linkage between resistance and the markers. The initial screening consisted of 96 SSR markers [*barc* (Song et al., 2002), *gwm* (Röder et al., 1998), *cfd* (Guyomarc'h et al., 2002), and *wmc* (Gupta et al., 2002; Wheat Microsatellite Consortium-http://wheat.pw.usda.gov/ ggpages/SSR/WMC)] that were evenly distributed across the D genome chromosomes according to the 2004 Chinese Spring wheat consensus map (Somers et al., 2004). This is based on findings that all non-rye derived RWA resistance genes map to the D genome (Liu et al., 2001, 2002, 2005; Ma et al., 1998; N. Lapitan, CSU Crop Genomics Program, USA, unpublished). SSRs that have been previously

linked to RWA resistance genes were included (Arzani et al., 2004; Liu et al., 2001, 2002, 2005; Miller et al., 2001; N. Lapitan, CSU Crop Genomics Program, USA, unpublished).

Primers were designed according to sequences published in GrainGenes (http://wheat.pw.usda.gov) with M13 sequence tags added to the 5' end of all forward primers (5'- ACG ACG TTG TAA AAC GAC + primer sequence -3') for fluorescence labeling purposes (Schuelke, 2000; Sun et al., 2009). A 10 µl reaction mix was used for PCR consisting of 50-100 ng DNA template, 2.5 mM $MgCl_2$, 0.2 nM dNTPs (Bioline, Boston, MA, USA), 1 x $(NH_4)_2SO_4$ buffer, 1 U *Taq* DNA polymerase (New England BioLabs, Ipswich, MA, USA), 0.2 µM reverse primer, and 0.1 µM each of M13-tailed forward primer and M13 universal primer labeled with either FAM (blue), NED (yellow), PET (red) or VIC (green) fluorescent tags. Fragments were amplified following the protocol described by Sun et al. (2009), using a PTC-200 Thermo Cycler with a 384-well block (MJ Research, Inc., Waltham, Massachusetts, USA). PCR products were multiplexed for detection by pooling four samples with different fluorescent labels to a final volume of 12 µl with 0.06 µl GeneScan-500 LIZ ® size standard (Applied Biosystems, Carlsbad, CA, USA) and 9.94 µl Hi-Di™ Formamide (Applied Biosystems, Carlsbad, CA, USA). The pooled marker fragments were analyzed on the ABI 3730 Genetic Analyzer (Applied Biosystems, Carlsbad, CA, USA) by the USDA Central Small Grain Genotyping Laboratory (Manhattan, KS, USA). The fragments were visualized and scored using GeneMarker v1.6 software (SoftGenetics, LLC, State College, PA, USA).

Linkage Map Development

The markers and RWA resistance from PI 626580 were tested for agreement with the expected Mendelian 1:2:1 genotypic segregation ratio using chi-square (χ^2) goodness-of-fit tests. The resistance gene was also tested for its fit to the 3:1 phenotypic segregation ratio expected for Mendelian inheritance of a single dominant gene. Genetic linkage between the molecular markers and resistance was determined using JoinMap v4.0 (van Ooijen and Voorrips, 2001) with a LOD threshold score ≥ 3.0. To account for potential crossover interference, the Kosambi mapping function was employed (Kosambi, 1943).

Quantitative assessment of polymorphic markers and RWA symptoms

The quantitative nature of symptom assessment suggested that the qualitative linkage mapping approach may not be the most appropriate method. Therefore, to determine whether or not the arbitrary class assignment based on chlorosis and leaf rolling scores was an appropriate method for the determination of genetic linkage between the molecular markers and RWA resistance, a quantitative trait loci (QTL) approach was also employed. A single-factor ANOVA using proc GLM was conducted for QTL detection using SAS v9.2 (SAS, 2008).

RESULTS

Inheritance of RWA resistance in PI 626580

Twenty-six days following RWA biotype 2 infestation, the resistant parent PI 626580-4 exhibited a highly resistant response with an average chlorosis score of 2.0 (on the 1-9 scale) and leaf rolling of 1.0 (on the 1-4 scale). This was contrasted by the severity of the susceptible response displayed by 'Yuma', the susceptible parent. On average, 'Yuma' plants were dead or unrecoverable with chlorosis and leaf rolling scores of 9.0 and 4.0, respectively. The F_2 individuals were assigned a phenotypic class (homozygous resistant, heterozygous, or homozygous susceptible) based on the number of resistant and susceptible individuals observed in the $F_{2:3}$ families. Homozygous resistant and susceptible designations were only given to F_2 individuals when all (or all but one) of the plants in the $F_{2:3}$ family were given the corresponding designation. Segregation for resistance among the 154-F_2 individuals was tested for conformation to the expected Mendelian 1:2:1 (R:H:S) genotypic and 3:1 (R:S) phenotypic segregation ratios for monogenetically controlled resistance, using chi-square (χ^2) goodness-of-fit tests. Among the 154 individuals, a ratio of 8:96:50 was observed which did not fit the genotypic 1:2:1 ratio ($P<0.001$) and instead exhibited significant segregation distortion that favored the paternal, 'Yuma' allele (Table 3.1). However, phenotypic segregation did fit the 3:1 ($P=0.100$) ratio suggesting adherence to the inheritance model for a single dominant gene (Table 3.1). Therefore, it is proposed that this new RWA resistance gene be designated *Dn10*.

Segregation of trichome densities by phenotypic class

Based on the Shapiro-Wilk test for normality, trichome densities were not normally distributed, therefore the data were log transformed by using the natural log of the densities. The log transformed data followed a normal distribution and were subject to the comparative ANOVA. Trichome densities of the homozygous resistance, heterozygous, and homozygous susceptible phenotypic classes were not significantly different (Table 3.2) and therefore, no additional investigation into the role of trichome density in the RWA biotype 2 resistance conferred by PI 626580 was conducted.

Microsatellite marker linkage mapping

Of the initial 96 D-genome specific SSR markers, *Xbarc214* and *Xgwm437* produced amplicons (237 bp and 124 bp, respectively) polymorphic between the parents and between resistant and susceptible bulks, associating with resistance from PI 626580-4. Since *Xbarc214* and *Xgwm437* have been mapped near the centromere on 7DS (GrainGenes-http://wheat.pw.usda.gov; Röder et al., 1998; Somers et al., 2004; Song et al., 2005a), 27 additional SSRs and 33 ISBPs, located near the centromere of 7DS (Paillard et al., 2003; H. Simkova, Institute of Experimental Botany Czech Republic, unpublished; Somers et al., 2004), were screened among the parents and bulks. Two SSRs, *Xgwm473* and *MS1* (the latter are alternative primers for the *Xcfd68* locus) (H. Simkova, Institute of Experimental Botany Czech Republic, unpublished), were polymorphic between the parents and between the bulks. They both amplified fragments (*Xgwm473*=244 bp and *MS1*=251 bp) associated with resistance from PI 626580-4 and were tested, along with *Xbarc214* and *Xgwm437*, in the entire F_2 population.

All four markers were co-dominant producing amplicons that segregated in coupling with resistance from PI 626580-4. Markers *Xbarc214*, *Xgwm437*, and *Xgwm473* followed the 1:2:1 segregation ratio, but segregation distortion was observed for *MS1* and it was therefore excluded from linkage map analysis (Table 3.3). A genetic linkage map for *Dn10* and the three linked markers, *Xbarc214*, *Xgwm437*, and *Xgwm473*, was constructed using JoinMap v4.0 (Figure 3.2). The markers were mapped in a single linkage group with a LOD score of 10. All three markers were proximal to *Dn10* with the nearest marker, *Xbarc214*, a genetic distance of 12.9 cM away. *Xgwm473* mapped at 16.0 cM from *Dn10* and *Xgwm437* was the furthest away at 19.2 cM. The order of the three SSR markers linked to *Dn10* is consistent with the Wheat-Composite 2004-7D map published on GrainGenes (http://wheat.pw.usda.gov) and the high density consensus map of 7D published by Somers et al. (2004). Both the composite and consensus maps show *Xbarc214* as the most distal marker of the three, *Xgwm437* as the most proximal and *Xgwm473* located in the middle. However, the cM distance between the markers varies from map to map and depends heavily on the backgrounds used in the mapping populations (GrainGenes-http://wheat.pw.usda.gov; Somers et al., 2004).

Quantitative evaluation of RWA resistance and linked SSR markers

The phenotypic segregation ratios and the quantitative nature of symptom assessment suggested that the qualitative linkage mapping approach may not be the most appropriate method. Therefore, to determine whether or not the arbitrary class assignment based on chlorosis and leaf rolling scores was an appropriate method for the determination of genetic linkage between the molecular markers and RWA resistance, a QTL approach

was also employed. Results of the single-factor QTL ANOVA provided information on the relative importance of the QTL in expression of chlorosis and leaf rolling with the percent of total phenotypic variance for the trait (% R^2) accounted for by the marker. The marker with the lowest *P* value and highest R^2 is thought to be closest to the QTL.

The single-factor QTL ANOVA supported the categorical phenotypic class assignments given to the $F_{2:3}$ families (Table 3.4). All three markers were highly associated with the QTL controlling both traits in this near-centromeric region of 7DS ($P< 0.0001$), as was expected since the markers were determined to be linked to the RWA gene found in PI 626580-4. *Xbarc214* was the highest contributor to phenotypic variance for both the chlorosis and leaf rolling QTL (R^2=64.6 and 64.8%, respectively). *Xgwm437* was determined to be the furthest away from the resistance QTL by contributing 60.8% of chlorosis variation and 60.7% of the variation observed in leaf rolling. As with the JoinMap results, *Xgwm473* was shown to be between *Xbarc214* and *Xgwm437*, contributing 63.1% of the symptom variance for chlorosis and 64.2% for leaf rolling.

DISCUSSION

Based on resistance to RWA biotypes 1 and 2 and evidence that resistance conferred by PI 626580 was potentially unique, the objectives of this study were to determine the inheritance of resistance and chromosomal location of the resistance gene, as well as identify linked molecular markers. Additionally, the role of trichome density in RWA resistance conferred by PI 626580 was assessed. Trichome density can be involved in aphid resistance by acting as a physical and/or biological deterrent (via exudation of allelochemicals) (Walling, 2008; Webster et al., 1994). However, this does not appear to be the case with resistance in PI 626580, since there was no difference in trichome density among the three phenotypic classes.

Dn10 is located near the centromere on the short arm of chromosome 7D. Several other RWA resistance genes have been mapped to 7D: *Dn1*, *Dn2*, *Dn5*, *Dn6*, *Dn8*, *Dnx*, and the unique CI 2401 resistance gene (Du Toit et al., 1995; Heyns et al., 2006; Liu et al., 2001, 2002, 2005; N. Lapitan, CSU Crop Genomics Program, USA, unpublished). Aphid resistance genes often occur as clusters within a specific chromosomal region (Dogimont et al., 2010) and Liu et al. (2005) suggested the potential presence of such a RWA gene cluster on 7D, near the centromere. *Dn1*, *Dn2*, *Dn5*, *Dnx*, and resistance genes found in four wheat plant introductions are thought to be in a cluster (or allelic) based on their tight linkage to each other and to *Xgwm111* (Liu et al., 2001, 2005; Miller et al., 2001).

There is the possibility of two more *Dn*-genes, *Dn6* and one of the resistance genes found in CI 2401, being located in the same cluster. *Dn6* was mapped by Liu et al.

(2002, 2005) on 7DS only 3 cM from *Xgwm111*, with the most recent study placing *Dn6* proximal to the marker. The unique (non-*Dn4*) resistance gene found in CI 2401 was mapped on 7DS, proximal to *Xgwm111* by 3.2 cM and flanked by both *Xbarc214* (0.8 cM) and *Xgwm473* (1.2 cM) (N. Lapitan, CSU Crop Genomics Program, USA, unpublished; H. Simkova, Institute of Experimental Botany, Czech Republic, unpublished). *Dn2* (on 7DS), in addition to its linkage to *Xgwm111*, is most closely linked to *Xgwm437* (Liu et al., 2001; Miller et al., 2001). This current study has shown that *Dn10* has linkage with *Xgwm214* and *Xgwm473* (similar to CI 2401) and *Xgwm437* (similar to *Dn2*). *Xgwm111* did not, however, show polymorphism between the parents or the resistant and susceptible bulks in this study. Whether or not *Dn10* is at the same locus or allelic to any of these other *Dn*-genes has yet to be determined, but it's possible that *Dn10* is a part of this 7DS cluster of RWA resistance genes.

Despite the segregation distortion that resulted in genotypic deviation from the expected 1R:2H:1S ratio, the F_2 population did fit the expected phenotypic 3R:1S segregation ratio. Therefore, it was concluded that the resistance of PI 626580 is conferred by a single dominant gene, provisionally designated as *Dn10*. Segregation distortion is the deviation of observed genotypic frequencies in segregating populations from those expected with inheritance of a single dominant gene and is often a problem encountered in mapping populations (Lu et al., 2002; Song et al., 2005b). There are several factors that can lead to this distortion, including pollen-tube competition, preferential fertilization, and zygotic selection (Song et al., 2005b). Competition among gametes due to the expression of specific gametophyte genes within a gamete and/or

genetic differences in the pollen have been attributed to nonrandom fertilization and deviation from Mendelian segregation ratios (Faris et al., 1998).

Segregation distortion has been reported in many crops, especially cereals such as rice (*Oryza sativa* L.), sorghum (*Sorghum bicolor* L.), maize (*Zea mays* L.), barley (reviewed by Lu et al., 2002) and wheat (Faris et al., 1998; Zhang and Dvorak, 1990). It has also been an issue with mapping populations involving RWA *Dn*-genes (Du Toit et al., 1995; Heyns et al., 2006; Liu et al., 2002). Non-Mendelian segregation of *Dn5* (and a possible allelic resistance gene found in a wheat plant introduction) was observed by Heyns et al. (2006) and Du Toit et al. (1995). Arzani et al. (2004) found significant segregation distortion of *Dn4*. This supported previous reports by Ma et al. (1998) and Liu et al. (2002) of significant genotypic distortion of *Dn4* and the closely linked marker *Xgwm106*, as well as segregation distortion (though not statistically significant) of *Dn6* in an F_2 population.

High levels of segregation distortion have been found on the D genome of wheat and its D genome progenitor (*Aegliops tauschii* Coss.), especially with chromosomes 1D, 3D-5D and 7D (Faris et al., 1998; Zhang and Dvorak, 1990). Interestingly, *Dn5* and *Dn6* are located on 7D and *Dn4* was mapped to 1D (Heyns et al., 2006; Liu et al., 2002). Faris et al. (1998) reported that a major region of distortion occurred near the centromere of *A. tauschii* chromosome 7D, based on an excess of homozygous paternal and heterozygous genotypes and a large deficiency in homozygous maternal genotypes. Of the five chromosomes exhibiting segregation distortion, 7D was the only one skewed towards paternal alleles (Faris et al., 1998). This distortion favoring the heterozygous and

homozygous paternal genotypes has also been seen in maize and cotton (*Gossypium spp.*) (Lu et al., 2002; Song et al., 2005b). Therefore, it's possible that the F_2 genotypic segregation distortion favoring the paternal 'Yuma' allele that was observed in this study was due to the close proximity of *Dn10* to the centromere on 7DS. However, the markers proximal to *Dn10* did not show the same distortion, so further research will be needed to clarify this issue.

It is also possible that there were more homozygous resistant F_2 lines than were scored as such due to incomplete penetrance or expressivity of the gene, resulting in a lack or reduction of resistant phenotypes observed among the $F_{2:3}$ families. The genetic background and modifier genes therein can play a major role in the full expressivity of a dominant gene and the phenotypes of the individuals that carry it (Junghans et al., 2003). Skewed phenotypic ratios for pest/pathogen resistance have been linked to the degree of penetrance for a variety of species including eucalyptus (*Eucalyptus psidii*), wild tomato species (*Lycopersicon peruvianum*), and wheat (Junghans et al., 2003; Rosello et al., 1998; Van der Westhuizen et al., 1998). This may be the case for this RWA resistance gene as well. Effectiveness of RWA resistance has been reported to vary depending on the background into which *Dn*-genes are bred (Botha et al., 2010; Van der Westhuizen et al., 1998). Van der Westhuizen et al. (1998) reported that glucanase enzyme activity during RWA infestation depended heavily on whether *Dn1* was present in 'Tugela', 'Betta', or 'Molopo' wheat cultivars and mentioned that overall effectiveness of RWA resistance was also background-dependent in field testing. It is not known how *Dn10* interacts within the 'Yuma' background, so whether or not incomplete penetrance is

responsible for the skewed segregation ratios via misclassification of phenotypic classes needs to be investigated further. Of course, there is the possibility of misclassification due to non-RWA related symptoms being mistaken for aphid-induced chlorosis and leaf rolling.

Since the categorical designation of resistance or susceptibility assigned to the $F_{2:3}$ families was done based on quantitative scores of chlorosis and leaf rolling, we wanted to see whether a QTL approach would provide similar results as the monogenic linkage analysis conducted using JoinMap. Single-factor ANOVAs were employed to determine the orientation of the three linked markers with respect to the QTL (in this near-centromeric region of 7DS) that influenced the RWA-induced chlorosis and leaf rolling symptoms. *P*-values for all three SSR markers were the same ($P < 0.0001$), so the R^2 values were the determining factor. Whether examining marker contribution to the QTL for chlorosis or leaf rolling, the order of the markers was the same with that closest to the QTL being *Xbarc214*, followed by *Xgwm473* and *Xgwm437* (furthest from both QTL). Thus, the results of the single-factor ANOVA were consistent with that of the linkage map derived using the phenotypic classes. This indicates that the cut-off values of chlorosis and leaf rolling used to assign resistant (chlorosis ≤ 4; leaf rolling ≤ 2) and susceptible (chlorosis ≥ 5; leaf rolling ≥ 3) designations to the $F_{2:3}$ families was appropriate for qualitative genetic linkage analysis. Therefore, future RWA mapping studies can consider these designations to be fairly accurate representations of total symptom development for monogenic linkage map development.

The location of the potentially novel, single dominant resistance gene *Dn10* was mapped near the centromere on the short arm of chromosome 7D. This study identified three *Dn10*-linked SSR markers, *Xgwm214*, *Xgwm473*, and *Xgwm437* at 12.9 cM, 16.0 cM, and 19.2 cM, respectively (Figure 3.2). SSRs lend themselves to high-throughput analysis; therefore these markers could provide an advantage in breeding wheat cultivars with improved RWA resistance (Guyomarc'h et al., 2002; Somers et al., 2004). However, further research should be conducted to identify markers with tighter linkage to *Dn10*, since the closest marker (*Xbarc214*) was 12.9 cM proximal to *Dn10*. With the development of new SSRs and ISBP markers in the region surrounding *Xbarc214* (H. Simkova, Institute of Experimental Botany Czech Republic, unpublished), the opportunity to identify more closely linked markers, including those distal to *Dn10*, is a tangible possibility in the near future. This will increase the likelihood of success of using marker-assisted selection for *Dn10*, including the potential of pyramiding with other *Dn*-genes, in wheat breeding programs interested in improved Russian wheat aphid resistance.

TABLES

Table 3.1 Phenotypic and genotypic segregation ratios and the results of the chi-square analysis for Russian wheat aphid resistance among $F_{2:3}$ families derived from a cross between PI 626580-4 and 'Yuma'.

Segregation	**Observed ratio**	**Expected ratio**	χ^2	***P* value**
phenotypic [t]	104:50	116:39	4.58	0.10
genotypic [t]	8:96:50	39:77:39	32.29	9.76E-08

[t] Chi-square values were based on a 3R:1S expected Mendelian phenotypic segregation ratio for a single dominant gene.

[t] Chi-square values were based on a 1R:2H:1S expected Mendelian genotypic segregation ratio for a single dominant gene.

Table 3.2 Trichome densities and standard errors for the classes phenotypic response to RWA biotype 2 infestation. Means followed by the same letter are not significantly different at the 0.05 probability level.

Phenotypic class	Density	Log density
resistant	372.1 ± 45.8[a]	5.87 ± 0.12[a]
heterozygote	346.9 ± 14.9[a]	5.78 ± 0.04[a]
susceptible	358.1 ± 21.8[a]	5.80 ± 0.06[a]

Table 3.3 Segregation of the polymorphic microsatellite (SSR) markers among 154-F_2 individuals derived from a cross between PI 626580-4 and 'Yuma'.

Marker	Observed ratio (R:H:S)[t]	χ^{2} [t]	*P* value
Xbarc214	39:70:45	1.74	0.42
Xgwm473	44:67:43	2.61	0.27
Xgwm437	44:64:45	4.10	0.13
MS1(cfd68)	16:93:44	17.37	1.69E-04

[t] R=homozygosity for the PI 626580-4 allele, H=heterozygous containing both R and S alleles, S=homozygosity for the 'Yuma' allele.

[t] Chi-square values were based on a 1:2:1 expected Mendelian genotypic segregation ratio for a single dominant gene.

Table 3.4 Results of the single-factor ANOVA for QTL detection conducted using the chlorosis and leaf rolling symptoms assessed for Russian wheat aphid resistance in wheat. The genetic distances in cM, as determined by the categorical linkage map constructed by JoinMap v4.0, are included for comparative purposes.

Symptom	Marker	Distance from *Dn10* (cM)	*P* value	R^2 (%)
chlorosis	*Xbarc214*	12.9	<0.0001	64.7
	Xgwm473	16.0	<0.0001	63.1
	Xgwm437	19.2	<0.0001	60.8
leaf rolling	*Xbarc214*	12.9	<0.0001	64.8
	Xgwm473	16.0	<0.0001	64.1
	Xgwm437	19.2	<0.0001	60.7

FIGURES

Figure 3.1 The collection site of the Iranian wheat landrace accession PI 626580 (modified from www.irantour.org).

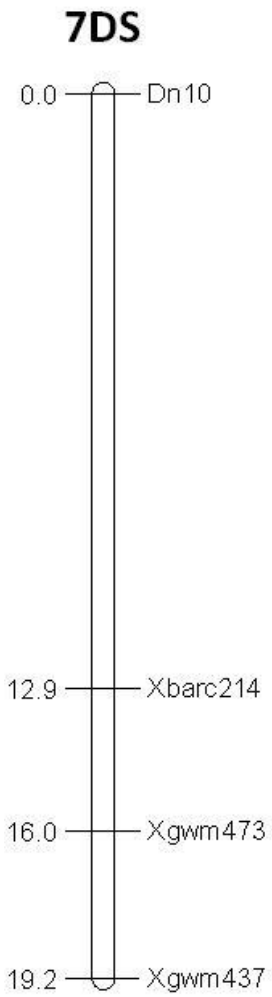

Figure 3.2 Linkage map of wheat chromosome 7DS containing the Russian wheat aphid resistance gene *Dn10* produced using JoinMap v4.0. Genetic distances are in centiMorgans (cM).

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