Virus-Induced Gene Silencing Suggests (1,3;1,4)- β -glucanase Is a Susceptibility Factor in the Compatible Russian Wheat Aphid–Wheat Interaction

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The Russian wheat aphid (RWA), *Diuraphis noxia* (Kurdjumov), is a significant insect pest of wheat (*Triticum aestivum* L.) and has a major economic impact worldwide, especially on winter wheat in the western United States. The continuing emergence of new RWA biotypes virulent to existing resistance genes reinforces the need for more durable resistance. Studies have indicated that resistance in previously susceptible plants can be produced by knock-down of susceptibility genes or other genes involved in host plant susceptibility. Therefore, investigation into genes involved in compatible RWA–wheat interactions could be a feasible approach to achieving durable RWA resistance. The objective of this study was to test whether silencing (1,3;1,4)- β -glucanase, previously observed to be highly induced in susceptible compared with resistant wheat during aphid infestation, would confer resistance to a susceptible wheat genotype. Barley stripe mosaic virus–mediated virus-induced gene silencing was employed to test whether (1,3;1,4)- β -glucanase is involved in the susceptible reaction of ‘Gamtoos-S’ (GS). Controlled infestation with U.S. biotype RWA2 was done to assess aphid reproduction and host symptom development. Aphids on (1,3;1,4)- β -glucanase-silenced plants reproduced less per day and had longer pre-nymphipositional periods than those on control GS plants. Furthermore, the (1,3;1,4)- β -glucanase-silenced plants exhibited less chlorosis and greater dry weight compared with GS. Aphid reproduction and host plant symptom development showed linear relationships with (1,3;1,4)- β -glucanase transcript levels. Our results suggest that (1,3;1,4)- β -glucanase is required for successful infestation by the RWA and may be a susceptibility factor that could be exploited as a potential target for RWA resistance breeding.

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 United States (Burd et al. 2006; Haley et al.

2004; Weiland et al. 2008). Direct and indirect damage due to RWA in the western United States alone exceeded \$800 million within the first 7 years after its discovery in 1986 (Morrison and Peairs 1998). RWA feeds on host phloem sap via intercellular insertion of its stylet into plant sieve tubes (Miles 1999; Will and Van Bel 2006). During feeding, aphids inject watery saliva containing effectors that either induce or suppress plant defense responses (Rodríguez and Bos 2013). Lapitan and associates (2007) demonstrated that injection of protein extract from RWA induced the development of susceptible symptoms in susceptible—but not resistant—plants. Compatible RWA–host interactions induce a series of host 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 those of most chewing insects in that they closely resemble pathogen infection (Halbert and Stoetzel 1998). It has been suggested that the RWA–host interaction follows a resistance (*R*)-gene-mediated, gene-for-gene model conferring race- or biotype-specific resistance (Botha et al. 2005; Flor 1971; Miles 1999; Ohtsuki and Sasaki 2006). To date, 12 named *R* genes have been identified; however, few confer resistance to the most virulent U.S. biotype designated as RWA2 (Haley et al. 2004; Porter et al. 2005), 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). However, investigations elucidating susceptibility factors are increasing in number and provide potential targets for breeding of resistance to various diseases and pests (De Almeida Engler et al. 2005; Pavan et al. 2010).

Susceptibility genes (*S* genes) tend to have a dominant mode of action in the host. In the presence of the corresponding virulence or effector gene products, *S* genes may elicit compatible interactions in an inverse gene-for-gene system and 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 using *Arabidopsis* mutants that prevented powdery mildew (*Blumeria graminis*) establishment and growth (De Almeida Engler et al. 2005). Loss-of-function mutations in *PRM6*, a pectate lyase-like gene, exhibited strong

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*The e-Xtra logo stands for “electronic extra” and indicates that two supplementary figures and two supplementary tables are published online.

resistance to powdery mildew independent of host defense activation, cell death, or presence of an *R* 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 also been identified in wheat, mostly from its compatible interactions with *Pyrenophora tritici-repentis* causing tan spot (Faris and Friesen 2005; Friesen and Faris 2004; Friesen et al. 2003), and *Stagonospora nodorum* causing leaf blotch (Friesen et al. 2008). More recently, Liu and associates (2013) have identified *Mayetiola destructor susceptibility-1* (*Mds-1*) as a gene contributing to susceptibility of wheat to the Hessian fly (*M. destructor* (Say)).

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

In a recent study comparing global gene expression profiles of resistant ('Gamtoos-R' [GR]) and susceptible ('Gamtoos-S' [GS]) wheat genotypes during infestation with RWA2, several genes were identified to be significantly upregulated in susceptible plants compared with resistant plants (Botha et al. 2010). Whether these genes provide resistance in susceptible plants upon silencing is not known and can be tested using virus-induced gene silencing (VIGS). VIGS is a post-transcriptional gene silencing technique that takes advantage of the double-stranded RNA-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). VIGS employing *Barley stripe mosaic virus* (BSMV) as a vector has mainly been a reverse genetics tool to study plant disease defense responses (Cakir et al. 2010; Dagdas et al. 2009; Scofield et al. 2005; Yuan et al. 2011) and has recently been shown to be effective for examining insect resistance as well. Van Eck and associates (2010) used BSMV-mediated VIGS to investigate insect resistance in wheat and showed that wheat plants silenced for *WRKY53* had decreased resistance to RWA.

The goals of this research were to test whether VIGS-mediated silencing of a wheat gene that is upregulated in the susceptible genotype GS during aphid infestation is involved in the compatible interaction with RWA2 and whether resistance to RWA2 is conferred to GS when the gene is silenced.

RESULTS

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

A previous microarray study conducted by Botha and associates (2010) identified 103 genes that were induced by RWA2 in the susceptible genotype, GS, or repressed during RWA2 infestation of the resistant GR plants. Ten genes with the greatest fold difference between GS and GR at 5 h postinfestation (hpi) are shown in Table 1. Transcripts ranked first (Ta.10.2.S1_x_at) and eighth (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). Because there is no reference genome sequence for hexaploid wheat, it is unclear whether these transcripts correspond to alleles of the same gene or are homologs on different genomes or even duplicate copies on the same or different chromosomes (Lai et al. 1993). Gene *(1,3;1,4)*- β -glucanase was chosen for this study based not only on rank but also because there has been no previous association published about the relationship between this gene and susceptible insect responses. Increased *(1,3;1,4)*- β -glucanase transcript abundance in GS compared with GR at 5 hpi with biotype RWA2 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 polymerase chain reaction (RT-qPCR) (Bustin et al. 2009). Under noninfested conditions, *(1,3;1,4)*- β -glucanase transcript abundance in GR is approximately 66% of the level observed in GS (Fig. 1). However, once biotype RWA2 infestation begins, the expression in GR decreases relative to GS, with expression in GS being 97% more than GR (Fig. 1). This differential expression at 5 hpi supports the results of Botha and associates (2010).

(1,3;1,4)- β -Glucanase expression reduction resulting from VIGS.

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 construct compared with *(1,3;1,4)*- β -glucanase expression in the control treatments. 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-}; therefore, they were removed from all but the regression analyses, where they served as internal viral controls. Thus, there were four biological replications for all treatment groups in each additional analysis. By employing BSMV-mediated VIGS, 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 (Fig. 2). The viral control GS plants inoculated with the empty viral vector (GS + BSMV_{BG-}) exhibited *(1,3;1,4)*- β -glucanase transcript levels, similar to the susceptible GS checks ($P = 0.945$). This degree of silencing is comparable with other VIGS studies in wheat (Scofield et al. 2005; Van Eck et al. 2010).

Table 1. Top 10 candidate wheat genes ranked by change in expression in the susceptible 'Gamtoos-S' (GS) compared with the resistant 'Gamtoos-R' (GR)^z

Rank	Probe-set ID	Gene ID	Logfold expression change (GS/GR)
1	*Ta.10.2.S1_x_at	<i>(1,3;1,4)</i> - β -glucanase	2.7
2	Ta.14729.1.S1_at	cDNA clone	2.4
3	Ta.1120.1.S1_x_at	cDNA clone	2.3
4	Ta.20250.1.S1_at	cDNA clone	2.2
5	Ta.15173.1.A1_at	Hypothetical protein	2.0
6	TaAffx.53974.1.S1_at	cDNA clone	2.0
7	Ta.25334.1.A1_at	Putative trehalose-6-phosphate synthase	1.9
8	*Ta.10.1.S1_a_at	<i>(1,3;1,4)</i> - β -glucanase	1.8
9	Ta.16407.1.S1_at	Hypothetical protein	1.8
10	TaAffx.525.1.S1_at	cDNA clone	1.8

^z Analysis was performed on data from a previous transcript profiling study conducted by Botha et al. (2010); * indicates candidate gene for this study.

Investigation into potential nontarget silencing.

Potential nontargets of silencing were identified by subjecting the VIGS construct designed from wheat (1,3;1,4)-β-glucanases to an across-species BLASTn pairwise analysis and, based on the identities of these sequences (Supplementary Table S1), (1,3)-β-glucanases were considered the most likely nontargets. RT-qPCR was performed to assess potential silencing and no difference in the abundance of (1,3)-β-glucanase transcript levels was observed among the four treatment groups (P = 0.53) (Supplementary Fig. S1).

Transcript levels affect RWA2 fitness and reproductive ability.

To determine the effect of (1,3;1,4)-β-glucanase transcript levels on RWA2 reproduction, nymph production was recorded by monitoring foundresses in individual clip cages. Nymphs born to the foundress in each cage were counted and removed daily to estimate effects of silencing on reproduction. This ensured that all aphid counts were based on the same reproductive starting point. The number of nymphs born per day was averaged across four biological replications within each treatment (Table 2). Silencing of (1,3;1,4)-β-glucanase reduced nymph production to 0.92 nymphs day⁻¹, less than the 1.46 observed on GS plants (P < 0.0001) but still more than the 0.65 nymphs day⁻¹ observed on GR plants (P = 0.046) (Table 2).

Prenymphipositional 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). The start of reproduction took an average of 9.0 days in the silenced treatment, which was significantly longer than the 7.3 days on GS

(P = 0.003) (Table 2). The nature of this delay in reproduction was not investigated in this study; however, these PNP values were consistent with previous findings that first nymphs are usually generated in approximately 7 to 10 days (Hodgson and Karren 2008). The viral control was similar to GS for each of the RWA2 reproductive measurements (mean births per day, P = 0.097; and PNP, P = 0.317) (Table 2).

With respect to aphid reproduction, the silenced treatment exhibited increased resistance over the susceptible controls (GS and GS + BSMV_{BG-}) through reduced aphid reproduction. Compared with GS, the GS + BSMV_{BG+} treatment supported fewer aphid births per day and promoted longer prereproductive periods.

Reduced levels of (1,3;1,4)-β-glucanase expression improve plant phenotype during RWA2 infestation.

GS leaves subjected to the GS + BSMV_{BG+} treatment were visually more vigorous and less chlorotic than the other GS treatments (Fig. 3); therefore, chlorosis and leaf rolling scores, along with plant dry weight measurements were recorded to quantify these visual observations (Tables 2 and 3). Chlorosis and leaf rolling were assessed on scales of 1 to 9 and 1 to 4, respectively, using a scoring system modified from Collins and associates (2005a). The GS + BSMV_{BG+} treatment showed a reduction in chlorosis from that of the susceptible check, GS, of 7.0 to 2.5 (P < 0.0001), with scores similar to GR (2.0) (P = 0.521) (Table 2). Additionally, the presence of the BSMV vector

Fig. 1. (1,3;1,4)-β-glucanase transcript expression in ‘Gamtoos-R’ and ‘Gamtoos-S’ (GS) tissues collected at 0 and 5 h postinfestation (hpi) with biotype RWA2. Real-time quantitative polymerase chain reaction values were standardized across the four biological replications, amplified in triplicate, calibrated against uninfested GS (0 hpi), and normalized to *UBQ5* with relative quantification and efficiency correction. Standard curves were run for control genes in every experiment. Means with the same letter were not significantly different at the 0.05 probability level.

Fig. 2. Barley stripe mosaic virus (BSMV)-mediated virus-induced gene silencing efficiency as revealed by differences in (1,3;1,4)-β-glucanase transcript abundance relative to the susceptible ‘Gamtoos-S’ (GS) treatment, the resistant treatment ‘Gamtoos-R’ (GR), the viral controls (GS + BSMV_{BG-}), and the silenced treatment (GS + BSMV_{BG+}). Real-time quantitative polymerase chain reaction 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. Standard curves were run for control genes in every experiment. Means with the same letter were not significantly different at the 0.05 probability level.

Table 2. Aphid reproduction and plant phenotype data for each treatment (reported with standard errors)^x

Treatment	Mean births day ⁻¹	Chlorosis ^y	Prenymphipositional period (days)	Leaf rolling ^z
GS + BSMV _{BG+}	0.92 ± 0.10 a	2.50 ± 0.29 a	9.00 ± 0.00 a	4.0 ± 0.0 a
GS + BSMV _{BG-}	1.46 ± 0.11 b	6.75 ± 0.95 b	7.75 ± 0.48 b	4.0 ± 0.0 a
‘Gamtoos-S’ (GS)	1.67 ± 0.07 b	7.00 ± 0.41 b	7.25 ± 0.48 b	4.0 ± 0.0 a
‘Gamtoos-R’ (GR)	0.65 ± 0.04 c	2.00 ± 0.00 a	10.00 ± 0.00 a	2.0 ± 0.0 b

^x Means within a column followed by the same letter are not significantly different at the 0.05 probability level.

^y Chlorosis scores range from 1 = healthy plants with small hypersensitive lesions to 9 = dead or unrecoverable.

^z Rolling scores range from 1 = completely flat leaves to 4 = tightly rolled with leaf trapping.

alone had no effect on chlorosis, as seen by the lack of difference in chlorosis between GS (7.0 score) and GS + BSMV_{BG-} (6.8 score) ($P = 0.747$). Leaf rolling was unaffected by transcript reduction because no difference in leaf rolling scores were observed 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).

To assess effects of *(1,3;1,4)-β-glucanase* silencing and aphid feeding on plant growth, above- and belowground plant biomass was determined (Table 3). Aboveground biomass of the GS + BSMV_{BG+} treatment was lower than that of GR (6.45 and 11.93 mg plant⁻¹ plant, respectively); however, it was greater than the 2.97 mg plant⁻¹ obtained for both GS ($P = 0.044$) and the viral controls, GS + BSMV_{BG-} ($P = 0.044$) (Table 3). Silencing did not have a significant impact on root biomass ($P = 0.094$) (Table 3).

***(1,3;1,4)-β-Glucanase* transcript levels show linear relationship with both host plant phenotype and aphid reproduction.**

To assess the linear relationship between *(1,3;1,4)-β-glucanase* expression at 14 days post-RWA2 infestation (19 days postsilencing) and RWA2 reproduction and host symptoms, regression analyses were performed (Fig. 4). The strongest linear relationships were observed with mean number of RWA2 births per day ($R^2 = 0.814$, $P < 0.0001$) (Fig. 4A) and PNP ($R^2 = 0.723$, $P < 0.0001$) (Fig. 4B), with the latter having an inverse relationship with transcript abundance. However, significant relationships were also observed between *(1,3;1,4)-β-glucanase* transcript level and total plant biomass ($R^2 = 0.494$, $P = 0.0011$) and chlorosis ($R^2 = 0.600$, $P = 0.0002$), the former showing an inverse relationship (Fig. 4C and D).

DISCUSSION

The presence of certain host plant genes is required for induction of susceptible plant–pest or –pathogen interactions, suggesting that plants may be less susceptible (more resistant) when expression of these genes is knocked down (De Almeida Engler et al. 2005; Pavan et al. 2010). These susceptibility factors may function as negative regulators of plant defense mechanisms or as *S* genes required for growth and development of the pest or pathogen (De Almeida Engler et al. 2005; Pavan et al. 2010). We investigated whether silencing of *(1,3;1,4)-β-glucanase* gene expression in a susceptible genotype using BSMV-mediated VIGS would result in increased resistance to RWA2. Resistance to RWA2 was assessed based on aphid reproduction and host symptom development.

Wheat *(1,3;1,4)-β-glucanase* is related to one of two barley *(1,3;1,4)-β-glucanase* isoenzymes called EI. The barley 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 not been 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,b). EI expression increases significantly during extended exposure to darkness due to decreased leaf sugar levels (Roulin and Feller 2001; Roulin et al. 2002). 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 RWA2 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). Based on sequence similarity, it is likely that barley EI and wheat *(1,3;1,4)-β-glucanase* have functions similar to Gsn1 of rice (Akiyama et al. 2009; Roulin et al. 2002).

Incompatible RWA reactions may involve three different mechanisms: antibiosis, antixenosis, and tolerance (Painter 1958; Smith 2005). To test whether the reduction of transcript levels in the susceptible background was related to 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 (PNP). The *(1,3;1,4)-β-glucanase*-silenced GS plants showed a reduction in births per day and an extended PNP compared with control GS, each exhibiting strong linear relationships with transcript levels. This suggests that reduction of *(1,3;1,4)-β-glucanase* transcript abundance may affect the growth, development, and reproduction of the RWA2.

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 or probing through enhanced stylet progress (Cherqui and Tjallingii 2000; Giordanengo et al. 2010; Goggin

Table 3. Individual component and total plant dry biomass for the treatments (reported with standard errors)^z

Treatment	Biomass (mg plant ⁻¹)		
	Root	Aboveground	Total dry
GS + BSMV _{BG+}	1.65 ± 0.04 a	4.80 ± 0.09 a	6.45 ± 0.13 a
GS + BSMV _{BG-}	0.85 ± 0.02 a	2.13 ± 0.03 b	2.97 ± 0.05 b
'Gamtoos-S' (GS)	0.85 ± 0.01 a	2.13 ± 0.03 b	2.97 ± 0.04 b
'Gamtoos-R' (GR)	2.75 ± 0.04 b	9.18 ± 0.13 c	11.93 ± 0.17 c

^z Means within a column followed by the same letter are not significantly different at the 0.05 probability level.

Fig. 3. Phenotypes of one representative plant per treatment 19 days post-BSMV inoculation (14 days post-RWA2 infestation). **A**, 'Gamtoos-R' (GR), **B**, 'Gamtoos-S' (GS), **C**, GS + BSMV_{BG-}, and **D**, GS + BSMV_{BG+}. Both susceptible treatments (B and C) exhibited the leaf rolling and chlorosis symptoms characteristic of RWA2 infestation. The *(1,3;1,4)-β-glucanase* silenced treatment (D) exhibited leaf rolling similar to that of the susceptible treatments (B and C) but was significantly less chlorotic and produced greater biomass, similar to the resistant GR (A).

2007; Harmel et al. 2008; Ma et al. 1990; Madhusudhan and Miles 1993). Because 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 reprogramming of host plant machinery to benefit the growth and propagation of the pest or pathogen (Giordanengo et al. 2010; Goggin 2007). A well-characterized example of this phenomenon is a family

of rice susceptibility genes belonging to the sugar transporter SWEET genes that are induced by bacterial pathogen and lead to increased sugar efflux in the phloem (Chen et al. 2010). The increase in (1,3;1,4)- β -glucanase expression in RWA2-infested susceptible wheat genotypes may be the result of aphid Salivary elicitor manipulation of host gene expression, resulting in effector-triggered susceptibility. This is in contrast to (1,3)- β -glucanases that are involved in resistant plant-pest or -pathogen interactions, including responses to aphid feeding (Iglesias et al. 2000; Lapitan et al. 2007; Smith and Boyko 2007; Van

Table 4. Wheat (1,3;1,4)- β -glucanase primers

Accession	Purpose	Length (bp)	Primer sequence
Z22873.1/Z22874.1	Real-time quantitative polymerase chain reaction	156	5'-GCCGCTCATGGCCAACATCTAC-3' 5'-TAGAAGGCGTCCACGGTGGTGT-3'
	Virus-induced gene silencing	338	5'-CCAACGCCAGGATCTACAAC-3' 5'-TACGCATCTGAGCTGCCTCA-3'

Fig. 4. Linear relationships between (1,3;1,4)- β -glucanase transcript expression at 14 days post-RWA2 infestation (19 days post-BSMV inoculation) and **A**, average number of births per day; **B**, prenympopositional period (PNP); **C**, total dry plant weight; and **D**, chlorosis severity. GS = 'Gamtoos-S', GR = 'Gamtoos-R', and * indicates plants in the GS + BSMV_{BG+} treatment that were not silenced at time of tissue collection, included here as internal viral controls.

der Westhuizen et al. 1998; Will and Van Bel 2006). In fact, Lapitan and associates (2007) reported increased (1,3)- β -glucanase enzyme activity in resistant wheat genotype 94M370 (containing *Dn7*) compared with GS under RWA2-infested conditions.

In addition to aphid growth and reproduction, we investigated the effect of (1,3;1,4)- β -glucanase silencing on host symptom severity by assessing leaf rolling and chlorosis, as well as measuring plant biomass. Based on our results, transcript abundance influenced progression of and shared a direct linear relationship with chlorosis development but had no impact on leaf rolling. This is consistent with previous observations that leaf rolling and chlorosis appear to be independently regulated (Botha et al. 2005; Lapitan et al. 2007). Chlorosis resulting from RWA feeding has been shown to occur by way of chlorophyll catabolism that differs from that of natural senescence; however, 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). The decrease in aphid reproduction and, thus, aphid-wounded area on the (1,3;1,4)- β -glucanase-silenced plants might account for the decreased chlorotic severity; however, the specific role of (1,3;1,4)- β -glucanase in chlorosis development and the mechanisms resulting in the reduced chlorotic responses seen in this study were not investigated. With respect to leaf rolling, it has been suggested that either ion gradients are disrupted, the plasma membrane suffers altered permeability from aphid feeding, or both (Moran et al. 2002) and this could cause rapid cell expansion and turgor in the lower cells, upper cell collapse, and subsequent mature leaf rolling (Botha et al. 2005; Van Volkenburgh 1999). The candidate gene, (1,3;1,4)- β -glucanase, has not previously been shown to be associated with these mechanisms but additional research will be needed to understand the reason for the lack of effect that (1,3;1,4)- β -glucanase silencing had on leaf rolling in our study as it was not investigated during the course of this research.

Because highly infested plants exhibit significant reductions in biomass, we measured plant dry weight to obtain an estimate of plant tolerance (Burd and Burton 1992; Mirik et al. 2009; Voothuluru et al. 2006). Tolerance involves surviving infestation and allows for the increased production of plant biomass compared with that of susceptible genotypes (Painter 1958; Voothuluru et al. 2006). Our results demonstrated 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 levels in GS was insufficient to achieve the high biomass 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 (1,3;1,4)- β -glucanase-silenced GS plants showed greater above-ground and total biomass than the nonsilenced GS control. It is currently unknown whether the increase in biomass observed is directly related to biomass accumulation processes or an indirect result of reduced RWA2 numbers, allowing for allocation of energy toward plant growth and not nutrient manipulation by the aphid (Giordanengo et al. 2010), since equal aphid numbers were not maintained (Reese et al. 1994).

The linear relationship between aphid reproduction and (1,3;1,4)- β -glucanase transcript levels observed in this study suggests that susceptibility to RWA2 may be reduced by the suppression of (1,3;1,4)- β -glucanase expression. Since the presence of the *Dn7* *R* gene differentiates the GR and GS near-isogenic lines (NIL), it follows that suppression of (1,3;1,4)- β -glucanase may be one of the 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 (1,3;1,4)- β -glucanase, because suppression of its expression in the susceptible GS background was associated with a more RWA-resistant phenotype. How (1,3;1,4)- β -glucanase expression affects susceptibility is not yet known. It may mobilize carbohydrate nutrients into the phloem to be intercepted by the aphid or may destabilize cell wall structures to allow for increased feeding efficiency or both. A more detailed examination into the role of (1,3;1,4)- β -glucanase in RWA2 susceptibility will provide insight into how (1,3;1,4)- β -glucanase suppression can be used in the implementation of novel RWA2 resistance in wheat breeding programs.

MATERIALS AND METHODS

Candidate gene selection.

Microarray data published by Botha and associates (2010) were analyzed to identify genes upregulated in GS compared with GR, 5 hpi with biotype RWA2. The data were sorted based on the logfold change in expression of GS/GR. The highest logfold change ratios corresponded to transcripts with the greatest abundance in RWA2-infested GS relative to RWA2-infested GR. Candidate gene selection for this study was based on rank in GS/GR logfold expression change (Table 1). Identification of and sequence information for the candidate gene was obtained through the National Center for Biotechnology Information (NCBI) and HarvEST Web databases.

Silencing construct development and sequence analysis.

VIGS primers were designed using Vector NTI v10 (Invitrogen Corp., Carlsbad, CA, U.S.A.) to amplify a 338-bp fragment spanning a portion of the coding sequence and 3' untranslated region of wheat (1,3;1,4)- β -glucanase cDNA sequence accessions Z22874.1 and Z22873.1 (Table 4). Constructs were designed based on parameters described by Scofield and associates (2005) with the goal of silencing all endogenous copies of wheat (1,3;1,4)- β -glucanase. A similar basic local alignment search tool for nucleotide sequences (BLASTn) from NCBI (Zhang et al. 2000) was employed to investigate sequence similarity and pairwise identity of the (1,3;1,4)- β -glucanase VIGS construct region to published sequences for determination of potential off-targets for silencing.

Following transformation into a pGEM-T Easy Vector (Promega Corp., Madison, WI, U.S.A.), the VIGS PCR product was digested with *NotI* (New England Biolabs, Ipswich, MA, U.S.A.) and ligated into the *NotI*-digested BSMV γ genome plasmid, pSL038-1 (Cakir and Scofield 2008), immediately 3' to the γ b gene in the γ pSL038-1 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 γ a and γ b genes. Once orientation was determined, the cloned fragments were sequenced to verify the identity of the insert.

Plant materials and growth conditions.

NIL of hexaploid wheat cultivar Gamtoos wheat were used for all experiments in this study. GR is the RWA2-resistant line, containing the *Dn7* *R* gene, developed from the transfer of the 1BL.1RS wheat-rye (*Secale cereale* L.) translocation from accession 94M370 to the susceptible 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, respectively, also with six plants per treatment. Each plant served as an independent biological rep-

lication, because at no point were samples within a treatment pooled. Three seeds were planted in each 6-in. 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 light cycle of 14 and 10 h, with approximately 24°C days and 20°C nights, and light intensities between 1,100 to 1,400 $\mu\text{M m}^2 \text{ s}^{-1}$. Since *(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 among and within treatments.

BSMV inoculation and silencing.

The α , β , and γ RNA genome segments (both with and without the insert) were synthesized from cDNA using the mMessage mMachine T7 kit (Ambion, Austin, TX, U.S.A.) followed by RNeasy mini kit spin column purification (Qiagen, Hilden, Germany). Each segment of the tripartite BSMV genome was combined in a 1:1:1 ratio (1 μg each) and added to an FES buffer (0.1 M glycine, 1% [wt/vol] bentonite, 1% [wt/vol] celite, 0.06 M K_2HPO_4 , and 1% [wt/vol] tetrasodium pyrophosphate, 8.5 pH), used as the abrasive for viral access into the plants. Two separate BSMV inoculations were prepared: one for silencing (BSMV_{BG+}) and one as a viral control (BSMV_{BG-}). The BSMV_{BG+} consisted of α , β , and γ + *(1,3;1,4)-β-glucanase* insert while the BSMV_{BG-} contained γ without any insert along with the α and β genome segments. BSMV inoculation was conducted on 12-day-old seedlings, at the three- to four-leaf stage, by rub inoculation of the first and second leaves (Scofield et al. 2005). A plastic covering was placed over the plants for 24 h to provide a humid environment, suitable for viral penetration and replication, after which time the plastic was replaced by netting to prevent premature RWA2 infestation.

RWA2 infestation.

The RWA2 were collected by in Lamar, CO in 2003. The colony was reared on RWA2 seedlings of nonvernalized, RWA2-susceptible winter wheat in a Conviron E15 environmental chamber with 14 h of light at 21°C and 10 hours of darkness at 19°C. Seedlings were planted three times a week, with three weeks of vegetative growth prior to RWA2 transfer.

Five days after BSMV inoculation, the third leaf of each plant in the experiment was fitted with a custom clip aphid cage. Two apterous adult biotype RWA2 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 to investigate aphid reproductive responses (Randolph et al. 2008). Following leaf caging, each plant was mass infested with approximately 150 biotype RWA2 aphids that had been extracted via a soil sieve from greenhouse host plants (Collins et al. 2005b). This was done to assess plant symptom response. Reinfestation 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 h for 14 days to assess aphid reproduction (Randolph

et al. 2008). To estimate antibiotic effects, the number of days to foundress reproduction (the PNP), and the mean number of aphid births per day per foundress were recorded (Lazzari et al. 2009; Webster 1990). Upon conclusion of counting, aboveground plant biomass was collected and placed in Berlese funnels to dry the plant matter while removing remaining aphids (Randolph et al. 2007). The aboveground dry plant biomass was weighed for each plant. Roots were dried in an oven at approximately 43°C for 48 h after being rinsed, then weighed to determine dry root biomass. The above- and belowground plant weights were used to determine effects of aphid feeding on each of the components separately, and a total dry weight calculation was used to determine the effect on overall plant growth as a modified tolerance assessment (Voothuluru et al. 2006).

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 ranged from 1 = healthy plants with small hypersensitive lesions to 9 = dead or unrecoverable, while leaf rolling scores were 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.

At 14 days after RWA2 infestation (19 days post-BSMV inoculation), the clip cages were removed and the area immediately surrounding and including the caged area, approximately 3.5 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) with RNeasy mini kit purification, including on-column DNase I digestion postextraction (Qiagen).

Real-time qPCR.

RT-qPCR of RWA2-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 and associates (2010) transcript profiling study. cDNA was synthesized using the Transcriptor cDNA synthesis system (Roche, Indianapolis, IN, U.S.A.) from RNA samples collected from GS and GR plants prior to infestation and 5 hpi with biotype RWA2. RT-qPCR was conducted using 2 ng of cDNA and PerfeCTa SYBR Green SuperMix for IQ (Quanta Biosciences, Gaithersburg, MD, U.S.A.) on a Bio-Rad MyiQ iCycler (Bio-Rad, Hercules, CA, U.S.A.). Each sample was amplified in triplicate to achieve three technical replications per sample. RT-qPCR products were amplified with primers (Table 4) using the following iCycler protocol: 60 cycles of amplification involving 2 min of initial denaturation at 95°C, 15 s of denaturation at 95°C, and a 45-s annealing and extension step at 60°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. The samples were normalized to ubiquitin 5 (*UBQ5*) and calibrated to the mean expression of uninfested GS (0 hpi) using relative quantification with efficiency correction (Pfaffl 2001). Standard curves were run for control genes in every experiment.

For determination of *(1,3;1,4)-β-glucanase* and *(1,3)-β-glucanase* transcript abundance after BSMV-mediated VIGS, first-strand cDNA was synthesized using a blend of random hexamers and oligo (dT)s with the qScript cDNA synthesis kit (Quanta Biosciences) 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) on a Bio-Rad MyiQ iCycler (Bio-Rad). Each individual biological replication was amplified in triplicate to provide three tech-

nical replications per biological replication. *(1,3;1,4)-β-glucanase* RT-qPCR products were amplified with the same RT-qPCR primers used for the Botha and associates (2010) microarray validation (Table 4), and *(1,3)-β-glucanase* was amplified with the primers listed in Supplementary Table S2, both with the following iCycler protocol: 2 min of initial denaturation at 95°C, 60 cycles of amplification involving 15 s of denaturation 95°C, and a 45-s annealing and extension step at 60°C for *(1,3;1,4)-β-glucanase* or 57°C for *(1,3)-β-glucanase* and the reference gene *18S*. The transcript abundance of each individual biological sample was averaged across the three corresponding technical replicates (Willems et al. 2008). 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). Standard curves were run for control genes in every experiment. The melt curves from the RT-qPCR reactions showed single peaks (Supplementary Fig. S2), which verified that the RT-qPCR primers used in this study were gene specific in GR and GS cDNA.

Statistical analysis.

Normality of the aphid reproduction and plant phenotype data was determined by Proc UNIVARIATE using SAS v9.2 (SAS 2008). Significance of treatment comparisons was 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 days postinfection and the RWA2 reproduction and host symptom data collected (SAS 2008).

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www.ncbi.nlm.nih.gov/
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