

Development of a Virus-Induced Gene-Silencing System for Hexaploid Wheat and Its Use in Functional Analysis of the *Lr21*-Mediated Leaf Rust Resistance Pathway¹

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Virus-induced gene silencing (VIGS) is an important tool for the analysis of gene function in plants. In VIGS, viruses engineered to carry sequences derived from plant gene transcripts activate the host's sequence-specific RNA degradation system. This mechanism targets the RNAs of the viral genome for degradation, and as the virus contains transcribed plant sequence, homologous host mRNAs are also targeted for destruction. While routinely used in some dicots, no VIGS system was known for monocot plants until the recent report of silencing in barley (*Hordeum vulgare*) by barley stripe mosaic virus (BSMV). Here, we report development of protocols for use of BSMV to efficiently silence genes in hexaploid wheat (*Triticum aestivum*). The VIGS system was first optimized in studies silencing phytoene desaturase expression. Next, we used it to assay genes functioning in leaf rust resistance mediated by *Lr21*, which encodes a nucleotide binding site-leucine-rich repeat class resistance gene product. We demonstrated that infection with BSMV constructs carrying a 150-bp fragment of *Lr21* caused conversion of incompatible interactions to compatible, whereas infection with a control construct or one that silences phytoene desaturase had no effect on resistance or susceptibility. Additionally, silencing the *RARI*, *SGT1*, and *HSP90* genes, known to be required in many but not all nucleotide binding site-leucine-rich repeat resistance pathways in diverse plant species, resulted in conversion to compatibility, indicating that these genes are essential in *Lr21*-mediated resistance. These studies indicate that BSMV-VIGS is a powerful tool for dissecting the genetic pathways of disease resistance in hexaploid wheat.

Wheat is one of the most important sources of protein in the human diet. It is a staple for 35% of the human population and supplies approximately 20% of the calories consumed worldwide (<http://www.cymmt.org/>). Losses from pathogens and pests greatly impact wheat production. One of the most pervasive of these diseases is leaf rust, caused by *Puccinia triticina*, which over the course of human history has caused famines and ruined the economies of entire countries (Agrios, 1988). Currently, worldwide annual losses from leaf rust are estimated to be the equivalent of U.S. \$2 billion (National Agricultural Statistics Service, <http://www.usda.gov/nass>).

Plants have evolved potent surveillance and response systems that provide resistance to a diverse set of pathogens, including fungi, bacteria, viruses, nematodes, and insects. Despite this broad range of in-

truders, in most cases examined, the resistance pathways (R-pathways) that counter them are based on very similar molecular architecture. One of the best-characterized modes of disease resistance is known as gene-for-gene resistance (Flor, 1971), whereby resistance to a specific pathogen requires the presence of a particular allele of a plant resistance gene (R-gene) and a gene encoding its cognate elicitor in the pathogen. The vast majority of R-genes that have been isolated are known to encode proteins that contain a nucleotide binding site (NB) and Leu-rich repeat domains (LRR) and are designated as NB-LRR proteins. NB-LRR proteins recognize pathogen effector molecules, either directly or indirectly, and activate a signal transduction pathway that results in expression of a resistance response that typically involves rapid ion fluxes, production of reactive oxygen intermediates, and rapid localized cell death at the site of infection, known as the hypersensitive response.

Intense analysis of NB-LRR-mediated resistance in model plants has greatly expanded our understanding of the mechanisms of gene-for-gene disease resistance. One of the great hopes for this research was that it would make possible the transfer of useful modes of resistance to agriculturally important crops. However, several studies have indicated that it is unlikely that R-genes will retain function when transferred across wide species boundaries, an effect known as restrictive

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taxonomic functionality (Tai et al., 1999). Although significant progress has been gained from studying disease resistance in model dicot plants, improvement of disease resistance in wheat will require the analysis of resistance mechanisms and the isolation of genes encoding R-pathway components from wheat.

The genetic analysis of disease resistance in wheat and isolation of genes encoding components of R-pathways is greatly complicated by the fact that most cultivated wheat (>90%) is hexaploid, also called common or bread wheat (*Triticum aestivum*). In addition to difficulties stemming from genetic redundancy, the wheat genome is extremely large, containing approximately 16,000 Mb of DNA (Arumuganathan and Earle, 1991), 128 times larger than *Arabidopsis thaliana*; Gill et al., 2004). Although elegant cytogenetic stocks exist to aid in coarse-scale mapping of wheat genes, many resources common in model plant systems do not exist. Mutagenized diploid wheat collections are not available, and, since wheat is recalcitrant to T-DNA transformation, no T-DNA insertion libraries exist in diploid wheat that could be used to streamline gene isolation. No active wheat transposons have been characterized, and transgenic systems for transposon-based gene tagging are not widely available (McElroy et al., 1997). Despite these obstacles, at least three wheat R-genes, *Lr21* (Huang et al., 2003), *Lr10* (Feuillet et al., 2003), and *Pm3b* (Yahiaoui et al., 2004), have been isolated through strategies employing chromosome walking followed by complementation in transgenic plants to confirm gene function. Nonetheless, it is clear that new tools are needed to increase the efficiency of gene isolation and functional analysis in wheat.

RNA-induced gene silencing should be a very useful tool for gene identification and functional analysis in hexaploid wheat. While there are many different systems for triggering RNA-induced gene silencing, all of them involve a common initial step, the production of large quantities of double-stranded RNA (dsRNA) within cells. Accumulation of sufficient levels of dsRNA activates a host defense mechanism that targets all of the sequence within the dsRNA for cleavage into short (21–25 nucleotide) interfering RNAs. The short interfering RNAs become incorporated into the RNA-induced silencing complex, where they direct the degradation of any RNAs with sufficient sequence complementarity (Denli and Hannon, 2003). RNA-induced silencing is particularly appealing in polyploid organisms because its homology-dependent action permits silencing of any redundant copies that share at least approximately 85% nucleotide identity (Thomas et al., 2001).

Virus-induced gene silencing (VIGS) strategies have proven very useful in the analysis of gene function in dicot plants (Lu et al., 2003b; Burch-Smith et al., 2004), but the only demonstration of effective VIGS in a monocot has been the report of silencing barley phytoene desaturase (PDS) expression using barley stripe mosaic virus (BSMV; Holzberg et al., 2002;

Lacomme et al., 2003). BSMV is a positive sense, single-strand RNA virus with a tripartite genome, composed of the α , β , and γ RNAs (Petty et al., 1989). Fragments of transcribed sequences from the plant gene to be targeted for silencing are inserted into a DNA plasmid, from which the γ RNA can be synthesized by in vitro transcription. The plant cDNA fragment is cloned immediately downstream of the termination codon of the γ b open reading frame. BSMV is known to infect other grasses, including wheat. In this study, we have tested whether BSMV can trigger gene silencing in hexaploid wheat and, if so, whether it can be used to assess the functions of genes participating in wheat disease R-pathways.

RESULTS

Silencing PDS Expression in Hexaploid Wheat Varieties

Our initial tests of BSMV-VIGS in wheat targeted the *PDS* gene, which provides a convenient visual reporter for silencing. *PDS* is essential in the carotenoid pigment biosynthetic pathway, and suppression of its activity results in photolysis of chlorophyll, also referred to as photobleaching, in the affected tissues. Two of the BSMV γ RNA constructs carried a 185-bp fragment of the barley (*Hordeum vulgare*) *PDS* cDNA in either the sense (BSMV:PDS4) or antisense orientation (BSMV:PDS4as) inserted just 3' to the stop codon of the γ b open reading frame. The sequence of the barley PDS4 fragment is 96% identical to the corresponding region of common wheat *PDS* expressed sequence tag (EST) BG909124 and, therefore, well within the range of homology necessary for effective silencing (Holzberg et al., 2002). Greenhouse-grown Bobwhite wheat and Black Hulled barley plants were inoculated with 1:1:1 mixtures of in vitro transcripts synthesized from plasmids containing the wild-type BSMV ND18 α , and β RNAs and derivatives of the γ RNA that carried either no plant sequence (BSMV:00) or the barley PDS4 or PDS4as fragments.

Seven days after rub inoculating the first and second leaves of 7-d-old seedlings with BSMV:PDS4 or BSMV:PDS4as, evidence of photobleaching was first apparent in third and fourth leaves of the barley plants. Photobleaching also developed in wheat, but it was not detectable until 10 d after viral inoculation. No evidence of photobleaching was observed in the plants infected with BSMV:00. Interestingly, the mosaic symptoms and chlorosis characteristic of BSMV infection in barley were much less pronounced in wheat.

The extent of photobleaching is similar in both species; the primary area affected is the base of the third leaf, while areas throughout the length of the fourth leaf are often bleached (Fig. 1A). Photobleaching was very rarely observed in the fifth leaves of either species. While the photobleaching phenotypes are very similar in wheat and barley, the most striking

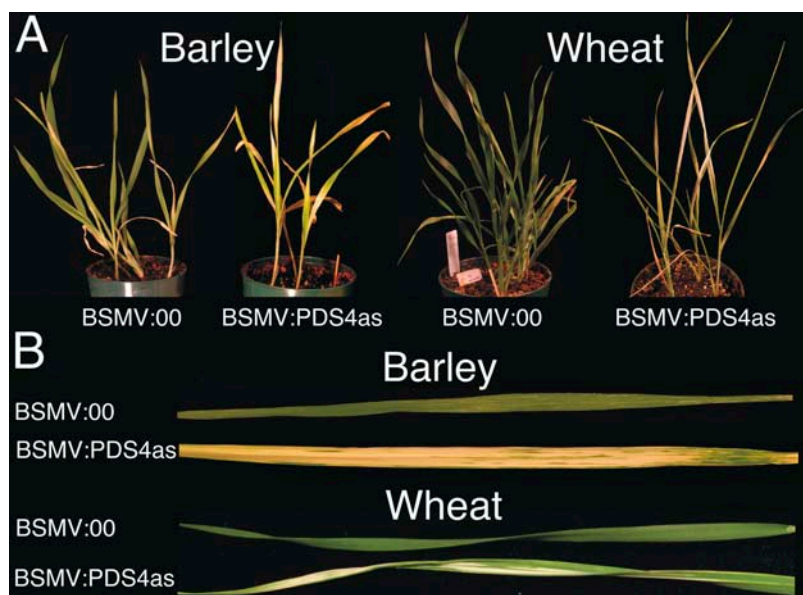


Figure 1. Silencing of *PDS* in barley and wheat by BSMV. Black Hulless barley and Bobwhite wheat were infected with in vitro transcribed RNAs representing the α , β , and γ RNAs of BSMV:00 or α , β , and γ PDS4as RNAs of BSMV:PDS4as 7 d after germination. These photographs were taken 12 d after infection and are representative of 10 different experiments. A, Whole plants; B, representative single leaves.

difference is that photobleaching in wheat often did not encompass the entire width of a leaf; photobleaching was often confined to narrow stripes that were parallel to the leaf veins (Fig. 1B). No consistent differences were apparent in the photobleaching resulting from the sense or antisense orientation of the PDS4 fragment (data not shown). Therefore, for simplicity we chose to use the antisense orientation of plant cDNA fragments in all subsequent work.

The BSMV:PDS4as photobleaching phenotype is robust and reliable and can be generated throughout the year in greenhouse-grown wheat; however, the affected leaf area was reduced and viral symptoms increased during the summer. This led us to explore the use of growth chambers. We found that growing the plants in a growth chamber set for 16-h light and operating at 25°C during the day and 20°C at night gave excellent results in VIGS experiments targeting *PDS* expression. In the course of our work, six additional hexaploid wheat varieties and one wheat-wheatgrass translocation line (Crasta et al., 2000) were tested with very similar results.

A derivative of the β RNA, in which the β a gene encoding the viral coat protein had been deleted ($\beta\Delta\beta$ a), was observed to generate more extensive areas of photobleaching in barley (Holzberg et al., 2002). Our tests of $\beta\Delta\beta$ a in barley confirmed these observations, but with significantly greater necrosis accompanying the photobleaching, whereas no advantage was observed in wheat (data not shown). The wild-type β RNA was used in all subsequent work.

Analysis of Sequence Length Required for Efficient Silencing of *PDS*

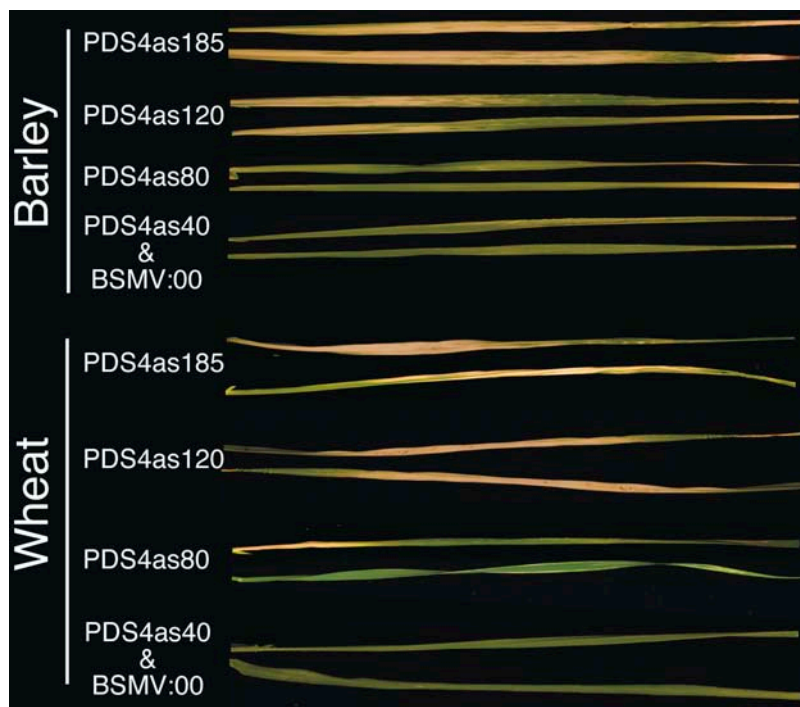
Previously, *PDS* fragments ranging in size from 1,215 to 185 bp (PDS4 and PDS4as) were compared

in their efficiency in silencing barley *PDS*, but no correlation between length and silencing efficiency was observed (Holzberg et al., 2002). We extended this analysis by making a more focused analysis of the lower limits of fragment size required for effective silencing by constructing a series of deletions from the 3' end of the PDS4as fragment. Silencing by derivatives of PDS4as with lengths of 120, 80, and 40 bp was compared in barley and wheat to the 185-bp PDS4as fragment (Fig. 2). In three replicate experiments, eight plants of each species were infected with each viral construct. A very clear decrease in photobleaching was observed in both wheat and barley when the PDS4as fragments less than 120 bp in length were tested. Only 21% of the wheat and 16% of the barley plants displayed any detectable photobleaching when the 80-bp PDS4as derivative fragment was tested. No photobleaching was observed in any of the plants infected with the 40-bp PDS4as derivative.

Time-Course Analysis of *PDS* mRNA Abundance when Targeted for Silencing by BSMV-VIGS

Our intention to use BSMV-VIGS for assessing the functional requirement for genes in resistance necessitates understanding the time course for establishment and maintenance of silencing. An appropriate interval of time can then be chosen between infecting with BSMV to initiate VIGS and the application of the pathogen to challenge the plant resistance system. To examine the time course of BSMV-VIGS, we employed quantitative real-time PCR (QRT-PCR) to measure the level of wheat *PDS* expression at 3, 7, 10, 13, 16, 18, and 21 d after inoculation with BSMV:00 or BSMV:PDS4as. Four plants were infected with BSMV:00 and BSMV:PDS4as for each time point. The third leaves of the plants for each treatment were pooled and total RNA

Figure 2. Comparison of silencing of *PDS* in barley and wheat by BSMV constructs carrying different length *PDS* sequences. Black Hulless barley and Bobwhite wheat were infected with in vitro transcribed RNAs from BSMV constructs in which the γ RNAs were engineered to carry a 185-bp fragment of *PDS* (PDS4as) or deletions of this fragment with lengths of 120, 80, or 40 bp. The third leaves were photographed 12 d after inoculation with BSMV. Ten plants were inoculated with each construct, and the experiment was repeated three times. The leaves shown are representative of all replicates. Silencing by BSMV:PDS40as was visually indistinguishable from BSMV:00.



was extracted. First-strand cDNA was synthesized from each pooled RNA sample using a mixture of random hexamers and oligo(dT) to prime synthesis. The level of endogenous *PDS* expression was determined by comparative QRT-PCR (Pfaffl, 2001) using 18S rRNA to normalize RNA amounts (Balaji and Anderson, 2005), and expression in the silenced sample is presented relative to *PDS* expression in leaves infected with the BSMV:00 control virus (Table I). Three biological replicates of this time-course study were performed.

This analysis indicated that silencing of *PDS* in the uninoculated third leaves was detectable as early as 3 d after inoculation with BSMV:PDS4as. Reduction of *PDS* expression by at least 60% was evident throughout the entire time course. Significant variation was evident between time points, and each biological replicate displays examples where the *PDS* message levels were found to be higher than the preceding or succeeding measurements. Given the variation in the visual appearance of photobleaching that is often observed, we believe that this fluctuation is likely to be a result of heterogeneity in the areas of leaf tissue affected by silencing. However, it is also possible that these variations reflect fluctuations in the level of silencing over time resulting from an unknown aspect of BSMV infection and silencing mechanisms.

Employing BSMV-VIGS to Identify Genes Required in Wheat Disease R-Pathways

Having demonstrated that BSMV can silence genes in various hexaploid wheat varieties and having defined the time course for the establishment and main-

tenance of BSMV-VIGS, we asked if this system could be used to assess the function of genes in a wheat disease R-pathway. The *Lr21* R-gene has been cloned and is known to encode a protein that is a member of the largest structural class of R-genes, the NB-LRR family (Huang et al., 2003). Three other genes, *RAR1*, *SGT1*, and cytosolic *HSP90*, have been found to function in many, but not all, of the NB-LRR R-pathways for which tools exist to permit functional analysis (Austin et al., 2002; Azevedo et al., 2002; Tor et al., 2002). Consequently, we attempted to test their requirement in *Lr21*-mediated resistance.

To make the silencing as specific as possible for *Lr21*, a 174-bp fragment comprised almost entirely from *Lr21* 3' untranslated sequence, designated *Lr21*

Table I. QRT-PCR time-course analysis of suppression of *PDS* expression by BSMV-VIGS in the third leaves of common wheat cv Bobwhite

Days after BSMV Infection	Relative <i>PDS</i> Expression ^a			Average Relative Expression ^a	SD
	Exp. 1	Exp. 2	Exp. 3		
3	0.393	0.357	0.363	0.379	0.019
7	0.508	0.417	0.243	0.389	0.135
10	0.120	0.330	0.616	0.355	0.249
13	0.097	0.392	0.011	0.166	0.200
16	0.298	0.50	0.654	0.332	0.304
18	0.048	0.452	0.444	0.315	0.231
21	0.163	0.164	NT	0.164	0.001

^aRelative *PDS* expression was calculated by dividing the expression value for *PDS* in silenced plants by the *PDS* expression level measured in plants infected with BSMV:00.

(4246-4420), was first chosen for cloning into the BSMV γ RNA. Genomic Southern-blot analysis showed one copy each of *RAR1* and *SGT1* genes in A, B, and D genomes of wheat (L. Huang, unpublished data). BLAST comparisons indicate that the longest wheat *RAR1* (BJ251120) and *SGT1* (BQ295431) ESTs share 93% and 97% homology to the barley transcripts, respectively. BSMV constructs were assembled using fragments of 412 bp and 592 bp, respectively, from the cloned barley *RAR1* and *SGT1* sequences (Shirasu et al., 1999; Azevedo et al., 2002). Very few data were available to guide the design of the HSP90 silencing experiment. Just one wheat EST, X98582, was found in the databases with significant homology to the barley cytosolic HSP90, and we had no wheat genomic Southern-blot data regarding the number of related sequences, so a 170-bp fragment was amplified from wheat cDNA with primers based on the X98582 EST sequence.

A wheat variety carrying the *Lr21* R-gene, WGRC7, and a susceptible line, Wichita (WI), were used in this analysis. Ten plants of each wheat line were inoculated with BSMV:00, BSMV:PDS4as, BSMV:Lr21, BSMV:RAR1, BSMV:SGT1, and BSMV:HSP90 7 d after germination. After an additional 8 d to permit establishment of VIGS, all of the plants were spray inoculated with *P. triticina* isolate PRTUS6, which is avirulent on WGRC7 and virulent on WI. Infection with BSMV:00 (Fig. 3A) or BSMV:PDS4as (Fig. 3B) had no effect on the normal resistance and susceptibility expected of WGRC7 and WI, respectively. However, when WGRC7 was infected with BSMV:Lr21 (4246-4420), BSMV:RAR1, BSMV:SGT1, or BSMV:HSP90, areas of susceptibility became apparent on approximately the distal most 10 cm of the third leaves of all the plants, indicating that the products of all four genes are required for *Lr21*-mediated resistance to PRTUS6 (Fig. 4). Infection of WI with each of these BSMV constructs had no observable effect on susceptibility (data not shown).

Comparative QRT-PCR was performed to confirm the silencing of each of the targeted genes (Table II). Sets of four WGRC7 plants were infected with BSMV:00, BSMV:Lr21 (4246-4420), BSMV:RAR1, BSMV:SGT1, or BSMV:HSP90. The third leaves of each set were collected 12 d after inoculation and pooled for RNA extraction. The expression level for each target gene was determined relative to the expression of the same gene in the BSMV:00-treated leaves. The degree of suppression determined for *RAR1* and *SGT1*, 54% and 83%, respectively, is similar to what had been determined for PDS; however, less suppression was measured for *Lr21* and *HSP90*.

In the case of *Lr21*, we have reason to believe that the level of suppression may actually be significantly greater than what was measured in this analysis. Because of the large number of NB-LRR genes in the wheat genome, a fragment from the *Lr21* gene that is comprised almost entirely of 3' untranslated sequence was selected for cloning into the BSMV γ RNA in an effort to enhance the specificity of *Lr21* silencing. *Lr21* is encoded on chromosome 1D of wheat (*Lr21D*);

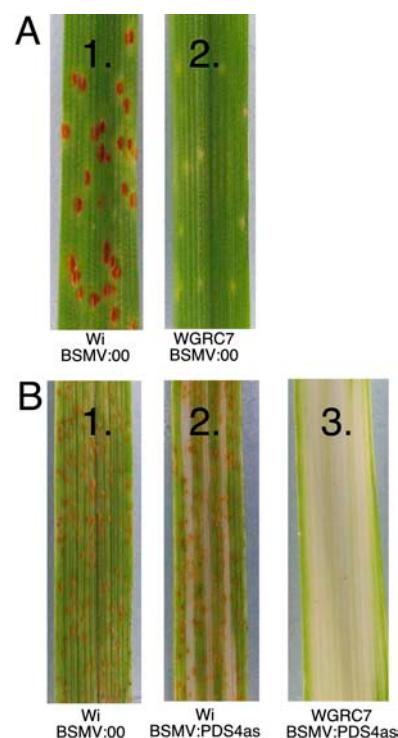


Figure 3. Leaf rust interactions of susceptible and resistant wheat after infection with BSMV:00 or BSMV:PDS4as. All plants were infected with the indicated BSMV constructs 7 d after germination and then spray inoculated with the avirulent *P. triticina* isolate PTRUS6 8 d after viral infection. The photographs were taken 10 d after inoculation with leaf rust and are representative of all leaves in two different experiments. A, The infection types of WI (1) and WGR7 (2) inoculated with BSMV:00. The necrotic spots in 2 are sites of *Lr21*-dependent hypersensitive response. B, In a separate experiment, infection type of WI inoculated with BSMV:00 (1) is shown on the left as a control. Infection with BSMV:PDS4as does not alter the infection type of WI (2) or WGRC7 (3).

however, homeoloci of *Lr21* are also present on chromosomes 1A and 1B, but they encode no known resistance specificities. After initiating these studies, a nullisomic1D-tetrasomic1A wheat line (missing 1D but having four copies of 1A and two copies of 1B chromosomes; Sears, 1954) was used to determine the sequences of *Lr21A* and *B* homeologs within the segment amplified in the *Lr21* QRT-PCR experiment. The A and B homeologous sequences were found to be identical to *Lr21D*. Therefore, if *Lr21A* and *B* are expressed, these QRT-PCR experiments should measure the combined expression of *Lr21A*, *B*, and *D* homeoloci. Comparison of the available sequences of *Lr21A*, *B*, and *D* did not identify a single polymorphic nucleotide in *Lr21D* that could be used to specifically measure its expression. *Lr21* homeologous sequences for the 3' coding regions and 3' untranslated regions of homeoloci on 1A and 1B are not yet available. PCR amplifications from the nullisomic1D-tetrasomic1A line, using primers based on the *Lr21D* sequence, have not been successful, suggesting that these regions may be divergent. Given these observations, it is possible that the expression of *Lr21D* was suppressed

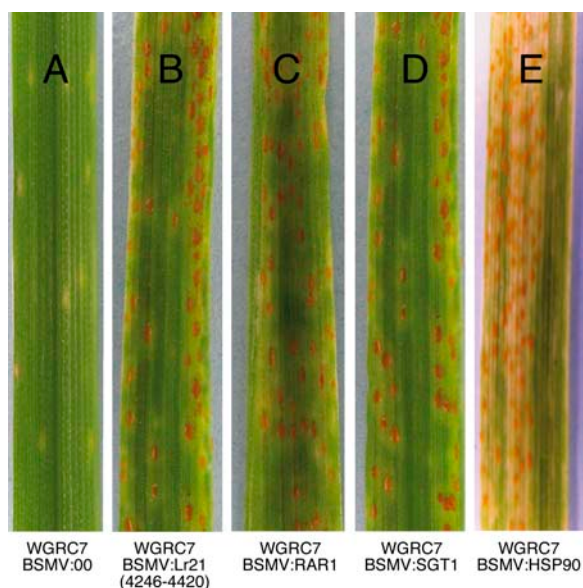


Figure 4. The effects of silencing *Lr21*, *RAR1*, *SGT1*, and *HSP90* on *Lr21*-mediated resistance. Ten plants resistant to PTRUS6 were inoculated with (A) BSMV:00, (B) BSMV:Lr21, (C) BSMV:RAR1, (D) BSMV:SGT1, and (E) BSMV:HSP90, 7 d after germination. All plants were spray inoculated with PTRUS6 8 d after viral infection, and leaf rust symptoms were photographed 8 d after fungal inoculation.

significantly, but this was masked by the expression of *A* and *B* homeologous sequences that were not silenced to the same extent.

To test this hypothesis, a second VIGS experiment using a conserved portion of *Lr21A*, *B*, and *D* homeologs was undertaken. A 219-bp fragment from the LRR domain of *Lr21* was cloned into the BSMV γ vector, and the resulting construct, designated BSMV:Lr21 (3493-3712), was used to silence *Lr21A*, *B*, and *D*. Silencing with this construct in WGRC7 resulted in similar conversion to susceptibility as seen previously with BSMV:Lr21 (4246-4420) (data not shown). As this silencing was performed targeting the conserved LRR portion of the gene, QRT-PCR probes derived from the 3' end of *Lr21D* that we hypothesized to be unique could be used to specifically measure *Lr21D* expression. As shown in Table II, these measurements indicate that *Lr21D* expression is suppressed by at least 80%, supporting our hypothesis. A similar explanation may apply to measurements of suppression for *HSP90*, but only one EST sequence is available for wheat *HSP90* at this time, so we know nothing about the sequences of other gene family members.

DISCUSSION

Here, we demonstrate that vectors based on BSMV can be effective reverse-genetics tools for the analysis of gene function in hexaploid wheat. Our initial tests focused on comparing the BSMV:VIGS systems in wheat and barley using the convenient photobleaching phenotype generated by silencing *PDS*. These

comparisons indicated that BSMV-VIGS gave largely similar results in barley and wheat. When the first and second leaves of barley and wheat were inoculated, large areas of photobleaching were observed in the third and fourth leaves of both species; however, in wheat photobleaching less frequently extends across the width of the leaf without interruption and often has a striped appearance. All of our *PDS* silencing studies in wheat were performed using derivatives of the barley *PDS* cDNA, which is 96% identical to the wheat EST sequence, with four divergent bases at positions 48, 66, 126, and 140 in the longest fragment tested (*PDS4* and *PDS4as*). Therefore, it is possible that these phenotypic differences may be due to the imperfect homology between the barley *PDS* VIGS fragment and the wheat genes targeted. Tests using the equivalent wheat *PDS* sequence will resolve this question.

In these studies, five different genes were targeted and successfully silenced in leaf tissue of wheat. Our long-term goal is to employ BSMV-VIGS to characterize the pathways of resistance to a number of pathogens of wheat, and, therefore, four of the genes that were silenced in this study were chosen for their likely involvement in gene-for-gene-mediated disease resistance. The *Lr21* NB-LRR R-gene serves as a positive control for BSMV-VIGS in the leaf rust assay because its function had been confirmed by complementation in transgenic wheat (Huang et al., 2003).

The remaining three genes tested, *RAR1*, *SGT1*, and *HSP90*, were chosen for study because they are among the small group of genes whose products have been demonstrated to be required for the function of multiple R-pathways in Arabidopsis, tobacco (*Nicotiana benthamiana*), tomato (*Lycopersicon esculentum*), and barley. *RAR1* has been shown to be required in about one-half of the R-pathways in which its function has been tested. *SGT1* was first identified as a *RAR1*-interacting protein in yeast two-hybrid screens. It is also required in a wide range of R-gene and non-host resistance mechanisms (Peart et al., 2002). Unlike *RAR1*, complete loss of *SGT1* function is lethal (Muskett and Parker, 2003); therefore, only transient silencing methodologies such as VIGS and biolistic bombardment of dsRNA can be used to assess *SGT1* functionality.

Table II. QRT-PCR analysis of *Lr21*, *RAR1*, *SGT1*, and *HSP90* expression during silencing by BSMV-VIGS

Gene	Relative Expression ^a	SD
<i>Lr21</i> (4246-4420)	0.859	0.034
<i>Lr21</i> (3493-3712)	0.171	0.050
<i>RAR1</i>	0.456	0.048
<i>SGT1</i>	0.170	0.069
<i>HSP90</i>	0.619	0.086

^aRelative expression was calculated by dividing the expression value determined for the target gene in silenced plants by the expression value of the same gene measured in plants infected with BSMV:00.

Recent studies have demonstrated that the cytosolic HSP90 plays an essential role in the *Rx*, *Pto*, *N*, *RPM1*, *RPS2*, and *RPS4* R-pathways (Hubert et al., 2003; Lu et al., 2003a; Takahashi et al., 2003; Liu et al., 2004; Zhang et al., 2004). HSP90 is known to function as a molecular chaperone that aids the correct folding of proteins in many biological systems, particularly molecules involved in signaling pathways (Picard, 2002). The chaperone functions of HSP90 are coordinated by cochaperone molecules, and evidence suggests that SGT1 and RAR1 may serve similar roles for HSP90. SGT1 has two structural motifs found in cochaperones of HSP90, the TRP and CS domains (Takahashi et al., 2003). Also, this model is strongly supported by the demonstration of pairwise interactions between HSP90, SGT1, and RAR1 (Hubert et al., 2003; Lu et al., 2003a; Takahashi et al., 2003) in various protein interaction assays. While much remains to be learned about the mechanisms of HSP90, RAR1, and SGT1, the evidence suggests that these three proteins act to stabilize the accumulation and to maintain R-gene products in the correct conformation for resistance signaling. The work described here indicates that *Lr21*-mediated resistance in wheat requires the expression of *RAR1*, *SGT1*, and *HSP90*.

We believe that BSMV-VIGS will be an excellent tool for the functional analysis of the *Lr21*-mediated R-pathway. The assay has been repeated many times under greenhouse and growth chamber conditions, and in each test silencing these genes resulted in a very clear conversion to susceptibility. A robust assay for loss of *Lr21*-mediated disease resistance will be a great advantage when we begin to screen for genes encoding novel *Lr21*-pathway components. VIGS screens for genes required in other R-pathways have been based on loss of the hypersensitive response as the primary criteria for identifying genes, but it was found that only a small fraction of genes identified in this way was actually essential for disease resistance (Lu et al., 2003a).

At the outset of our work, we were concerned that the physiological stresses associated with viral infection or defense responses to BSMV might interfere with our ability to observe the operation of the R-pathways that we are attempting to functionally dissect. However, it was noted that the symptoms associated with infection with the BSMV:00 control virus that contains no plant gene sequences were significantly less severe in wheat than in barley. This suggests that the background physiological changes associated with BSMV infection may be less severe in wheat, which would be a great benefit for work investigating disease resistance or other plant processes. Additionally, in our work with several different barley and wheat varieties, we observed that in contrast to the wheat lines, which were uniformly conducive to BSMV-VIGS, several barley lines supported BSMV-VIGS at clearly reduced levels. Variation in the susceptibility of different barley genotypes to BSMV infection has been reported (McKinney, 1965), and

resistance to BSMV has been an objective in some barley improvement programs (Sisler and Timian, 1956). This, together with our own limited experience, suggests that there may be less genotypic restriction in the application of BSMV-VIGS in wheat in comparison to barley.

It is interesting to note that the location of tissue that displayed susceptibility to *P. triticina* in our studies was not coincident with the area of maximum photobleaching in the *PDS* silencing experiments. Susceptibility developed in the distal 10 cm of the third leaves, while photobleaching most frequently appeared at the base of the third leaf and rarely extended into the tip region. The limitation of susceptibility to the leaf tip is a consequence of leaf growth that occurs during the 8 d between the application of *P. triticina* and the development of rust symptoms; the lower portion of the leaf was not exposed to the fungus because it had not formed at the time of fungal inoculation. However, in the case of *PDS*, while silencing occurs throughout the length of the third leaf, photobleaching is rarely observed near the tip. We believe this is best explained by the biological differences underlying these two phenotypes. *PDS* has an essential function in the production of carotenoid pigments, which are likely to be very stable once produced. Therefore, if *PDS* silencing is not established before these pigments are synthesized at the leaf tip, photobleaching will not appear there (Guo et al., 2003). Our results suggest that the gene products of *Lr21*, *RAR1*, *SGT1*, and *HSP90* are significantly less stable than carotenoid pigments. Therefore, even if silencing of these R-pathway components is not established as the tip of the third leaf forms, it is possible to observe silencing there at a later time, after silencing is established and prevents replacement of these gene products as they turnover.

Development of an effective VIGS system for wheat is a significant achievement. Conventional methods of gene isolation and confirmation of gene function are not easily accomplished given wheat's large genome with high ratios of physical to genetic distance, high genetic redundancy due to polyploidy, and its recalcitrance to most methods of transformation that involve regeneration. The steps required to utilize the BSMV-VIGS system are straightforward and, with the exception of inoculating plants with BSMV transcripts, not highly labor intensive. We anticipate that large-scale screens of hundreds of candidate wheat cDNAs will be possible with BSMV-VIGS.

MATERIALS AND METHODS

Construction of BSMV-Derived Vectors

The plasmids utilized in these experiments are based on the constructs described by Holzberg et al. (2002). The BSMV γ constructs utilized to evaluate the silencing efficiency of different length *PDS4* fragments were constructed as follows: Derivatives of the *PDS4as* fragment (Holzberg et al., 2002) with lengths of 120, 80, and 40 bp were generated by performing three PCRs

using pyPDS4 as a template. A common reverse primer, TATGCGCCGCC-TACTTTCAGGAGGATTAC, was used with the following forward primers: ATATTAATTAAACAATTTCATAAACCCCTGACG, ATATTAATTAATGATTGC-TCTAAACCGTTTTTC, and ATATTAATTAATAAAAAATGGCATTCTTGGATGG. The PCR products were digested with *NotI* and *PacI* and ligated into *NotI* + *PacI*-digested pyPDS4as (Holzberg et al., 2002). Silencing of *Lr21* and *HSP90* was performed using a γ RNA vector, pSS031-1, which was generated by digesting pyPDS4as with *NotI* + *PacI* and replacing the PDS4as insert with annealed oligonucleotides that form a unique *SmaI* site between the *NotI* and *PacI* cloning sites.

PCR Amplification of Plant Genes

The fragments used to silence *Lr21*, *RAR1*, and *SGT1* were generated by PCR amplification from plasmids containing the cloned genes. A 172-bp fragment of *Lr21* was amplified from the plasmid pLr21 with the forward primer GAACGAAGATGACGAACAA and reverse primer ATGAGCCGG-TACTAAAGGTC. A 412-bp fragment of barley (*Hordeum vulgare*) *RAR1* was amplified from plasmid pLK14 (Shirasu et al., 1999) using the forward primer ATATTAATTAAAGCAACCCACCAAAGTCAGC and reverse primer TATGCGGCCGCATCAGCATTGTGCCACCCCTTTG. A 592-bp fragment of barley *SGT1* was PCR amplified from the plasmid pCA110 (Azevedo et al., 2002) with the forward primer ATATTAATTAAGGAGAATGCACAGCCAA-CGGTAG and reverse primer TATGCGGCCGCCGTCCTTTCCCGACATC-TTTCC. To generate the fragment of common wheat (*Triticum aestivum*) *HSP90* that was used for silencing, total RNA was prepared from WRG7 and reverse transcribed as described below. A 170-bp fragment of *HSP90* was amplified from this cDNA using the forward primer GGCGCTGAGAACGACAC and the reverse primer AAACCGATACAGCGACCATCC.

In Vitro Transcription of Viral RNAs and Plant Inoculations

Capped in vitro transcripts were prepared from three linearized plasmids that contain the tripartite BSMV genome using the mMessage mMachine T7 in vitro transcription kit (Ambion, Austin, TX), following the manufacturer's protocol. These in vitro transcription reactions typically result in 1 to 1.5 $\mu\text{g}/\mu\text{L}$ final concentration of RNA. Plants were infected with BSMV using a modified protocol (Holzberg et al., 2002). One microliter of each of the in vitro transcription reactions for the α , β , and γ RNAs were combined and added to 22.5 μL of FES (Pogue et al., 1998). This mixture was then applied to plants by rub inoculation. The mixture was pipetted between the pinched thumb and first finger of a gloved hand. The base of the plant to be inoculated was then held with the other hand while the first leaf was gently squeezed between gloved first finger and thumb. The entire leaf surface was then coated with this mixture by sliding the gently pinched fingers from base to tip two times. Over the course of our studies, greater than 95% of the wheat plants that were inoculated with BSMV:PDS4as developed photobleaching.

RNA Extraction and cDNA Synthesis

Total RNA was extracted from plants using the TriZol (Sigma-Aldrich, Milwaukee, WI) protocol as given by the manufacturer. All RNA samples were digested with DNase I prior to synthesizing cDNA, using the TURBO DNA-Free kit as recommended by the manufacturer (Ambion). First-strand cDNA was synthesized using the I-SCRIPT kit (Bio-Rad, Hercules, CA), following the manufacturer's protocol.

Measurements of mRNAs by QRT-PCR

Expression of the genes targeted for silencing was quantified by comparative QRT-PCR. The measurements were performed in a Stratagene MX3000P QRT-PCR machine using the I-TAQ SYBR reagent kit (Bio-Rad). Four plants were grown and infected with each BSMV construct. The third leaves of each treated plant were harvested 12 d after infection, unless described otherwise. Leaves from each treatment were pooled and total RNA was prepared as described above. QRT-PCR was performed in quadruplicate for each RNA sample/primer combination. The amounts of RNA in each reaction were normalized using primers specific for 18S rRNA. The sequences used to detect each gene are as follows: 18S forward, GTGACGGGTGACGGAGAATT; 18S reverse, GACTAATGCGCCCGGTAT; PDS forward, TGTCTTTAGCGTG-CAAG; PDS reverse, GATGATTTCGGTGTCACT; HSP90 forward, CGACC-

AGCACGCTCACGAT; HSP90 reverse, GCGATGGTCCCCGAGGTTGT; RAR1 forward, ATGCGGTGCCAGCGAATA; RAR1 reverse, GGGTTGTCGTCGT-CGGTG; SGT1 forward, CAAGCTGGCAGTTAC; and SGT1 reverse, GCTT-TATGCATCGAAGGA. Suppression of *Lr21* using the *Lr21* (4246-4420) VIGS construct was measured with the following primers: *Lr21* forward, AGCC-CGGTCATGTGACTA, and *Lr21* reverse, GGAGCTGTTGGTTCGTGT. Suppression by *Lr21* (3493-3712) was assayed with the following primers: *Lr21*(2) forward, GAACGAAGATGACGAACAA, and *Lr21*(2) reverse, ATGAGCC-GGTACTAAAGGTC. In all cases, expression of the targeted gene is presented as the expression level in the silenced plant relative to expression of the same gene in plants infected with BSMV:00.

Plant Material and Growth Conditions

Barley cv Black Hulless and common wheat cv Bobwhite, WI, and WRG7 seeds were sown in 4-inch pots containing potting soil and placed under greenhouse conditions: 20°C to 25°C with supplemental light to provide daylengths equal to or greater than 11.5 h and watered as needed. Plants were also grown in a Conviron PGR15 growth chamber (Winnipeg, Canada) equipped with high-intensity discharge lamps, with light intensity ranging between 450 and 700 $\mu\text{mol m}^{-2} \text{s}^{-1}$, programmed for 16-h days with 25°C during the day and 20°C at night.

Puccinia triticina-Wheat Interaction Assays

Leaf rust resistance was evaluated on newly emerging leaves 8 d after viral inoculation. BSMV-infected plants were sprayed with *P. triticina* spores (isolate PRTUS6) using an air compressor and nozzle, then placed in a moist chamber at 15°C overnight. Infection type was documented and photographed 9 to 10 d after rust inoculation.

Availability of Biomaterials

Upon request, all novel materials described in this publication will be made available in a timely manner for noncommercial research purposes, subject to the requisite permission from any third party owners of all or parts of the material. Obtaining any permissions will be the responsibility of the requestor.

The use of any trademarked products does not constitute an endorsement by the U.S. Department of Agriculture, Agricultural Research Service.

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