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Article

BSMV-Mediated Assays for Oat Functional Genomics

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Abstract: Herbicide-resistant weed populations in numerous cropping systems pose a significant threat to global food security by reducing crop yields and increasing production costs. Multiple herbicide-resistant (MHR) *Avena fatua* L. (wild oat) plants with non-target site resistance (NTSR) mechanisms can express resistance to herbicides without previous exposure and are thus particularly troubling. Although precise molecular mechanisms for resistance to five different mechanisms of action are not clear, numerous transcription factors and stress-related proteins are preferentially subjected to constitutive post-translational modifications (PTMs) in MHR plants. Transient assays to quickly assess if changes to specific genes can change the MHR response will facilitate the development of new strategies to manage MHR weeds. Our study showed that the Barley Stripe Mosaic Virus (BSMV) mediated system can be an option for *A sativa* (cultivated oat) and *A. fatua* (wild oat) functional analysis. The inoculation efficiency of this system ranged from 48% to 100%. Its efficacy was demonstrated by successfully knocking down phytoene desaturase (PDS) expression and overexpressing green fluorescent protein (GFP), suggesting its potential for oat functional genomics.

Keywords: BSMV; VIGS; VOX; *Avena fatua*; *Avena sativa*; functional genomics

1. Introduction

Virus-induced gene silencing (VIGS) is an RNA-mediated reverse genetics technology with wide applications for analyzing gene function (1,2). VIGS is typically used to downregulate endogenous genes by exploiting plant post-transcriptional gene silencing processes. The first VIGS vector was adapted from a tobacco mosaic virus (TMV) (3), although most applications for members of the Poaceae use a barley stripe mosaic virus (BSMV)-derived vector (4). For example, VIGS has been successfully used in wheat (5), barley (6), and maize (7). The related use of BSMV-derived vectors for gene overexpression (VOX) also has been reported for wheat (8) and other members of the Poaceae. However, previous VIGS studies on hexaploid (*Avena sativa*) and diploid (*A. strigosa*) oats were not successful (9), and we are not aware of reports on the use of VIGS for *A. fatua*.

The widespread and intensive use of herbicides has been selected for resistant weed populations in numerous cropping systems, posing a significant threat to global food security by reducing crop yields and increasing production costs. Of all resistant species, multiple herbicide-resistant (MHR) *Avena fatua* L. (wild oat) populations have a disproportionate economic impact due to their worldwide presence and competition in small grain crops (10). MHR weeds with non-target site resistance (NTSR) mechanisms can express resistance to herbicides without previous exposure and are thus particularly troubling (11).We recently described MHR inbred lines of the pernicious weed *Avena fatua* (wild oat) that are resistant to at least 10 members of HRAC Mode of Action Groups (12) 0, 1, 2, 15, and 22 (13,14).

In addition to several disparate NTSR mechanisms, resistance co-segregates with constitutively elevated expression of genes with roles in stress response, xenobiotic metabolism, heat shock response, disease resistance, and transcriptional regulation (15). Additional comparisons of soluble

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proteins revealed that MHR plants contain constitutively elevated levels of proteins with functions in core biosynthetic pathways (glycolysis, Calvin cycle, C-2 glycolate pathway), stress response pathways (photorespiration, mannose-binding lectin, protein kinase), and stress-related transcription factors. Phosphoproteome and redox proteome analyses show that numerous transcription factors and stress-related proteins are preferentially subjected to constitutive post-translational modifications (PTMs) in MHR plants (16).

The functions of these candidate genes and their potential linkage with MHR merit further investigation. We propose that the use of VIGS and VOX can provide valuable insights into the molecular mechanisms of MHR, an idea first proposed for resistant species by Macgregor in 2020 (17). In *Alopecurus myosuroides* (blackgrass), loss and gain of function of the glutathione *S*-transferase AmGSTF1 successfully changed the MHR response to fenoxaprop (18). The current work demonstrates the successful knockdown of the *A. fatua* phytoene desaturase (*PDS*) and overexpression of a green fluorescence protein (*GFP*) gene in *A. fatua* and *A. sativa* using a BSMV-mediated system as a first step in manipulating MHR phenotypes.

2. Materials and Methods

2.1. Plant Material and Growth Conditions

Inbred MHR and herbicide susceptible (HS1) *A. fatua* lines were derived from field-selected populations as previously described (15,19), and a second inbred HS line (HS2) was derived from the nondormant *A. fatua* line SH430 used in seed dormancy research (20,21). Plants were grown in 10.2 cm square pots under a 16-hr photoperiod of natural sunlight supplemented with mercury vapor lamps (165 µmol m^{-2} sec^{-1}) at 25 ± 4 C. Growth medium was greenhouse soil mix [1:1:1 (by vol) Bozeman silt loam:Sunshine mix #12 (Sun Gro Horticulture, Inc., Bellevue, WA):perlite], plants were fertilized weekly with 100 ppm of Jack's Classic 20-20-20 All Purpose fertilizer, and watered as needed. Plants used for inoculations were tested at Zadoks (21) stages 10 to 12 (1 ½ leaf to 2 1/2 leaf stage, respectively).

2.2. Preparations of BSMV-RNAs

The pα, pβ, and pγ plasmid DNAs were linearized with restriction enzymes *Mlu*I, *Spe*I, and *Mlu*I, *respectively. The pγPDS and pγGFP were linearized with BssHII. Each reaction was conducted in a 20 µl volume* containing 2.5 µg plasmid DNA, 6 units of enzyme, 2 µl 10X enzyme buffer, and nuclease-free H_2O. All enzymes were from New England, Biolabs (Rowley, MA, USA). The reactions with *Mlu*I or *Spe*I were incubated at 37°C for 90 mins and then inactivated at 80°C for 20 mins. Reactions with *Bss*HII were incubated at 50°C for 3 hours and then heat-inactivated at 65 °C for 20 mins. The completion of linearization was checked side-by-side with the corresponding uncut plasmid via electrophoresis on 1% w/v agarose gel.

Linearized plasmid DNAs were treated with 40 units (1µl) RNase inhibitor (New England, Biolabs Inc) per 20 µl linearization reaction. *In-vitro* transcription was performed in a 20 µl reaction containing 2 µl T7 10X buffer, 1 µl of 20 U/µl T7 RNA polymerase (Sigma Aldrich), 2 µl cap / rNTP mix, 7 µl linearized plasmid, and 8 µl nuclease-free H_2O. The reaction was incubated at 37°C for 2 hours. Transcription reactions were examined on a 1% w/v agarose gel by sampling 1 µl of each reaction every 2 hours while incubation continued. We prepared 20 µl of each α, β and γ/PDS/GFP RNAs for 20 plants.

2.3. Inoculation Methods

We used the leaf-rub inoculation as described by Huang (2017) (22) to introduce RNAs of α, β, and γ/PDS/GFP (BSMV:00; BSMV:PDS and BSMV:GFP) into plants. *In vitro*-transcribed RNAs were combined in a 1:1:1 ratio, which amounts to 60 µl of total transcript for each of the 20 plants to be inoculated. We then added 440 µl FES buffer and mixed thoroughly on ice. Plants were inoculated with 23 µl RNA, which was rubbed on the second leaf between the index finger and thumb three

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times starting at the base and extending to the tip of the leaf while holding the plant stem steady with the other hand.

2.4. Green Fluorescence Imaging

GFP fluorescence images were captured with a confocal light microscope (Zeiss Axioscope 7) and photographed with the ZEISS Axiocam camera portfolio and ZEISS ZEN 2 core imaging software. Leaf samples showing visible viral symptoms at 14 dpi with BSMV:GFP and BSMV:PDS were viewed under a microscope using bright light and fluorescence.

2.5. Quantitative RT-PCR Analysis

Total RNA was isolated from leaf tissues of BSMV:PDS and BSMV:00 *inoculated plants* using the RNeasy plant mini kit (Qiagen, Valencia, CA, USA) and treated with RNase-Free DNase (Qiagen, Valencia, CA, USA) to remove DNA based on the manufacturer's instructions. The concentration of total RNA was assessed using 260/280 ABS measurements on a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, DE, USA). RNA integrity was checked using a 1% agarose gel at 120 volts for 30 mins of 1 µl RNA, 4 µl of water, and 1 µl loading buffer (98% formamide, 10 mM EDTA, 0.25% [w/v] bromophenol blue, and 0.2% [w/v] xylene cyanole) and stained with GelRed (Bio-Rad, Hercules, CA, USA).

Quantitative real-time PCR (qRT-PCR) was performed on a CFX 96 real-time PCR detection system (Bio-Rad, Hercules, CA, USA). The reaction contained 2 µl of a mixture of forward and reverse primers (1 pmol/µl), 10.25 µl of iScript One-Step SYBR Premix (Bio-Rad, Hercules, CA, USA), 2 µl of RNA (37.5 ng/µl), and 5.75 µl of RNase-free water. PDS gene expression was assayed using primers flanking the inserted region of the gene in the γPDS vector (Table 1). The housekeeping gene alpha glucan phosphorylase (GLUP) was used as a reference gene. The PDS silencing level is presented as the fold change of $2^{-\Delta\Delta Ct}$ relative to the abundance of the gene in BSMV:00 inoculated oats.

Table 1. Primers used in the study.

Name	Sequence	Tm (°C)	Size of the amplicon (bp)
AfPDSF1	TGGATAACTCGTCAGGGTTTATG	54.3	109
AfPDSR1	ACTGTTTCAGAGTGGATGGAAA	54.1	
GLUP F	CCTGGCAGTGAATTGTCTCAG	55.8	148
GLUP R	AAGTTTTCCTCTCCAACCTCC	54.6	

AfPDSF1, *A. fatua* phytoene desaturase forward primer; AfPDSR1, *A. fatua* phytoene desaturase reverse primer; GLUP F, *A. fatua* alpha glucan phosphorylase gene forward primer; GLUP R, *A. fatua* alpha glucan phosphorylase gene reverse primer.

2.6. Statistical Analysis

qRT-PCR data are presented as the means of absolute quantification number (Ct) ± standard deviation (SD) of three biological replicates. The data were subjected to t-tests using comparisons of mean PDS RNA abundance from BSMV:PDS- to BSMV:00-inoculated plants. Statistical differences between mean values were determined using standard errors in Microsoft Excel.

3. Results

3.1. Construction of Gamma (γ) Vectors for BSMV-Mediated Assays

The DNAs corresponding to the three sub-genomes of BSMV were cloned into three plasmids designated as pα, pβ and pγ, comprising respectively the tripartite genomes (α, β and γ) of BSMV (23). Two constructs, each with a 215-bp or a 300-bp fragment of the *A. fatua* PDS gene (Supplementary file 1) were inserted into the pγ-PCR cloning vector (24), (Figure 1A), and termed

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pγPDS. The GFP reporter gene was used to evaluate BSMV-mediated gene overexpression. The GFP coding sequence plus a 110-bp ribosome binding site (RBS) at the 5'end was inserted in an inverted orientation after the stop codon of the BSMV γ genome in the pγ-PCR cloning vector *(Figure 1B, supplementary file 2) and termed pγGFP.*

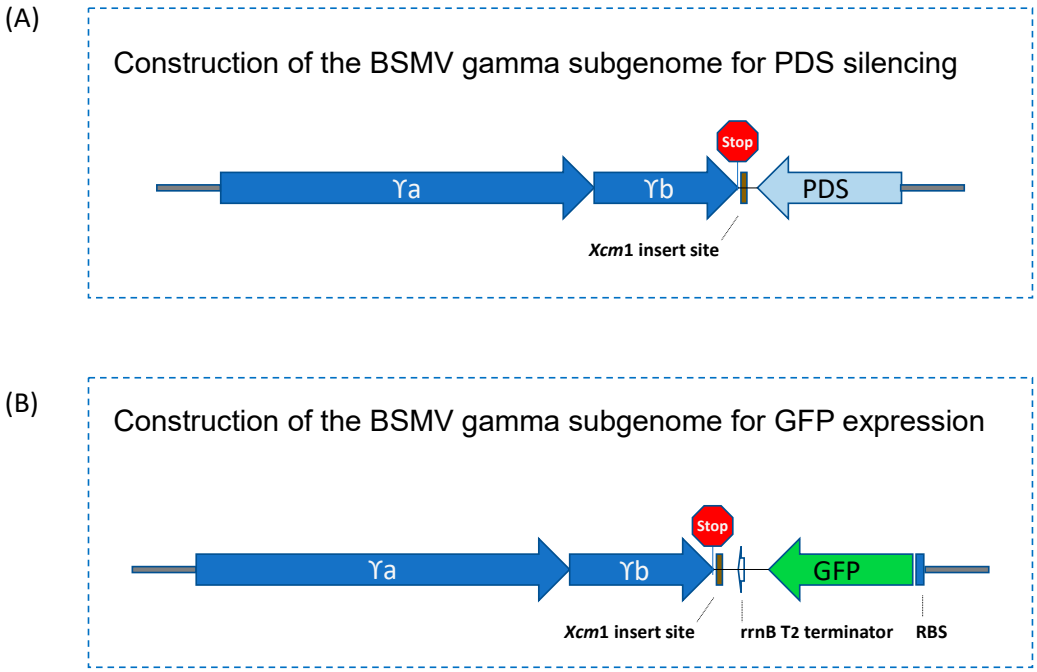

Figure 1. Construction of the gamma vector for BSMV-mediated assays. A: Construct for gene silencing; B: Construct for gene expression. rrnB: ribosomal RNA in Escherichia coil. RBS: ribosomal binding site.

3.2. BSMV-Mediated Gene Silencing in Oats

An essential requirement for using a virus-mediated assay in a plant is that the virus must infect the plant. To test if BSMV can infect oats and induce gene silencing, we rub inoculated the RNAs of the virus onto the second leaves of 20 plants from each of *A. fatua and A. sativa at the 2-leaf stage.* Five wheat plants were included as a positive control to confirm the quality of the experiments since we routinely use BSMV-mediated gene silencing assays for wheat functional analysis (5,25). For the silencing assay, we used PDS as a reporter gene because silencing of this gene gave a photobleaching phenotype. In-vitro synthesized RNA of α, β, and γPDS was mixed in a 1:1:1 ratio (BSMV:PDS) for inoculation, and an α, β, and γ mixture containing only the BSMV genome (BSMV: 00) was used as a control. Streak patterns were visible on BSMV-inoculated oat plants at 14 days post-inoculation (dpi) (Figure 2), suggesting BSMV could replicate in both *A. sativa* and *A. fatua.* However, there were significant differences in the time required and severity of viral symptoms between wheat and oat plants. Mosaic viral symptoms were evident within 9 dpi for wheat, but only after 14 dpi for oats. Further, symptoms were more severe on wheat than on oat plants (Figure 2). The efficiency rate of BSMV inoculation for *A. sativa* and *A. fatua* was around 50%, while the rate for wheat was 100% (Table 2).

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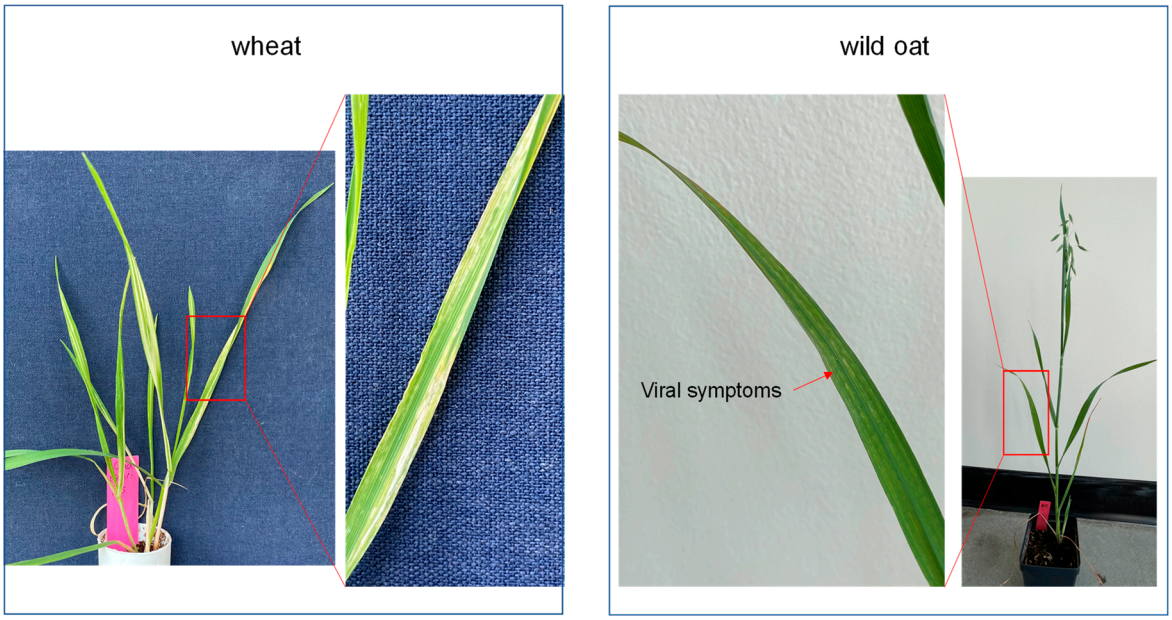

Figure 2. BSMV symptoms of wheat and wild oats at 14 days post-inoculation. Red arrows indicate the leaf after zooming in.

Table 2. Efficiency rate of BSMV inoculation.

Treatments	Lines	Efficiency rate (%)
BSMV:PDS	*A. fatua*	50
	A. sativa	48
	T. aestivum	100
BSMV:GFP	*A. fatua*	82
	A. sativa	66
	T. aestivum	100
BSMV:00	*A. fatua*	100
	A. sativa	50
	T. aestivum	100

Among BSMV:PDS inoculated plants, we observed a photobleaching phenotype in *A. sativa* (Figure 3) and *A. fatua* at 18 dpi, while no photobleaching was observed in plants inoculated with BSMV:00 (Figure 4). Photobleaching was rarely observed in leaves that emerged before inoculation for both *Avena* species.

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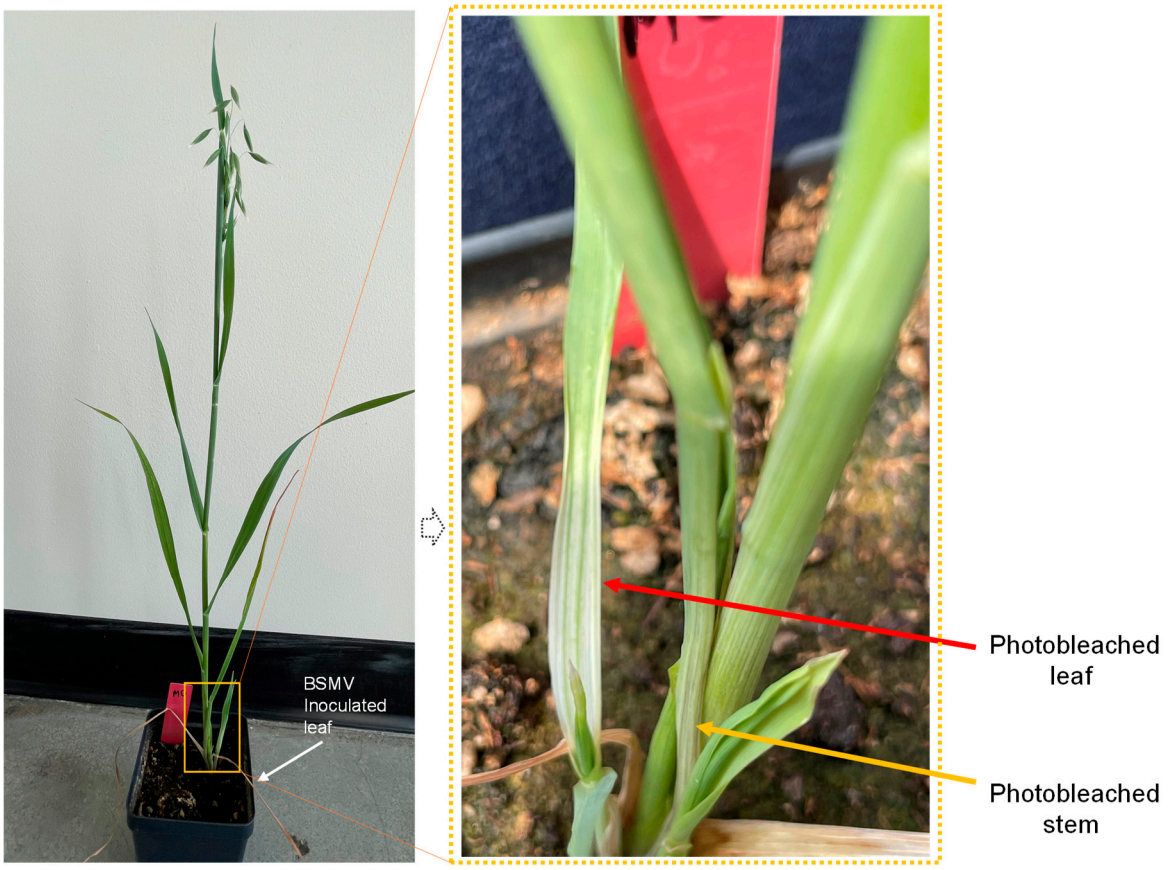

Figure 3. The photobleaching phenotype of *A. sativa* at 18 dpi with BSMV:PDS inoculation.

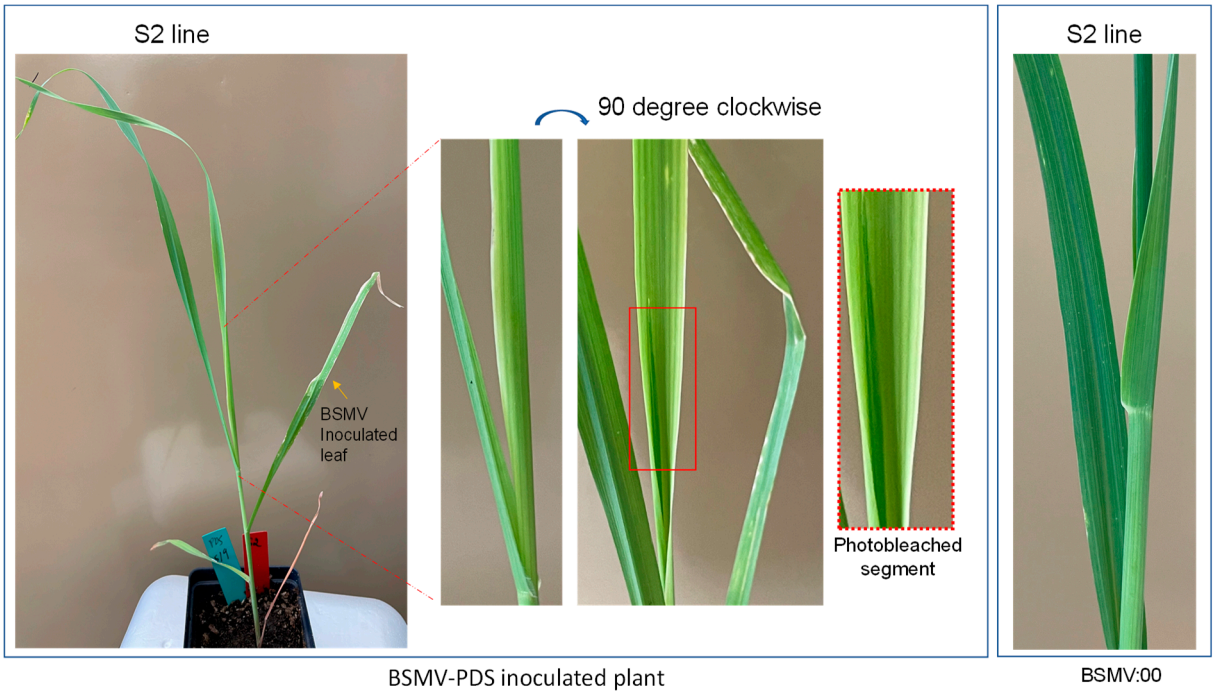

Figure 4. The photobleaching phenotype of *A. fatua* at 18 dpi with BSMV:PDS inoculation.

Three photobleached segments of *A. fatua* plants were sampled and tested for the PDS transcript abundance. The corresponding segments from three BSMV:00 inoculated *A. fatua* plants were used to reference the PDS transcript abundance level. The qRT-PCR revealed a significant reduction of

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PDS in the silenced segments, about 25% of the transcript level in the PDS silenced segments related to the non-silenced control (Figure 5).

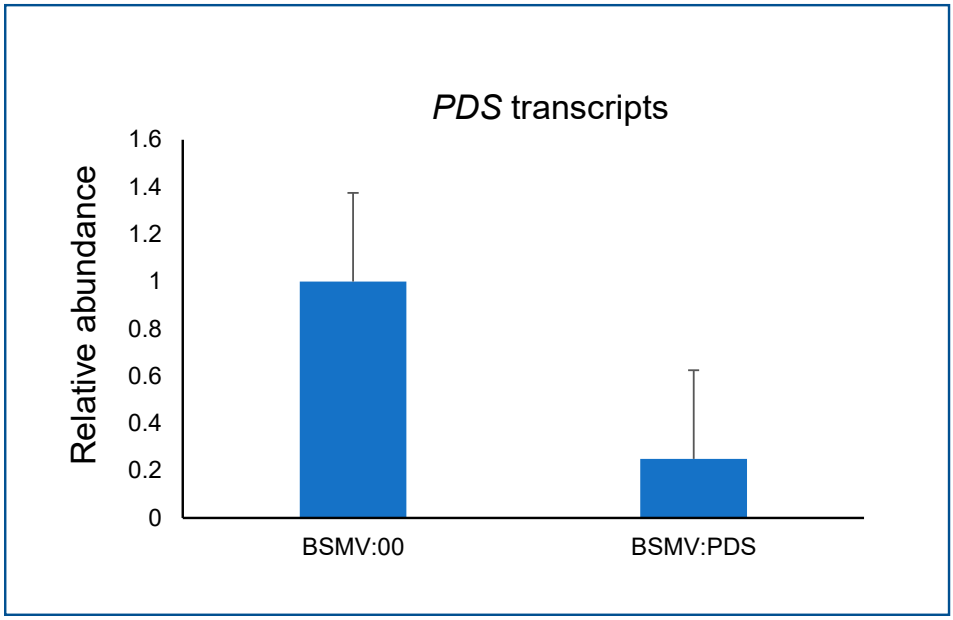

Figure 5. The relative PDS transcript abundance between BSMV:00 (non-silenced control) and BSMV:PDS inoculated plants.

Our results demonstrate that BSMV can infect two species of oats and induce their silencing machinery as an antiviral defense response. To our knowledge, this is the first report of BSMV-based VIGS in either species of oats.

3.3. BSMV-Mediated Gene Over-Expression in Oats

Having demonstrated that BSMV can replicate and activate silencing machinery in *A. fatua*, we further tested whether this system could be used to over-express a gene of interest. Similar to the silencing assay described above, *in-vitro* synthesized RNA of α, β, and γGFP was mixed in a 1:1:1 ratio as BSMV:GFP and used to inoculate 20 *A. sativa* and *A. fatua* plants at the 2-leaf stage. At 14 dpi, visible virus symptoms were observed, and symptomatic sections of leaves that emerged after inoculation were viewed under a fluorescence microscope in two different light settings. Under bright light, patches of viral symptoms were distinguishable as lighter green on leaves from plants treated with BSMV:GFP or BSMV:PDS (Figure 5). When the leaf sections were viewed under fluorescence with a green filter, green fluorescence signals were clearly visualized and overlapped with the patches of viral symptoms on leaves from the BSMV:GFP treated plants, but no/zero signals from the BSMV:PDS inoculated plants (Figure 6). This result demonstrated that BSMV could be used to overexpress a gene of interest in *A. sativa* and *A. fatua.*

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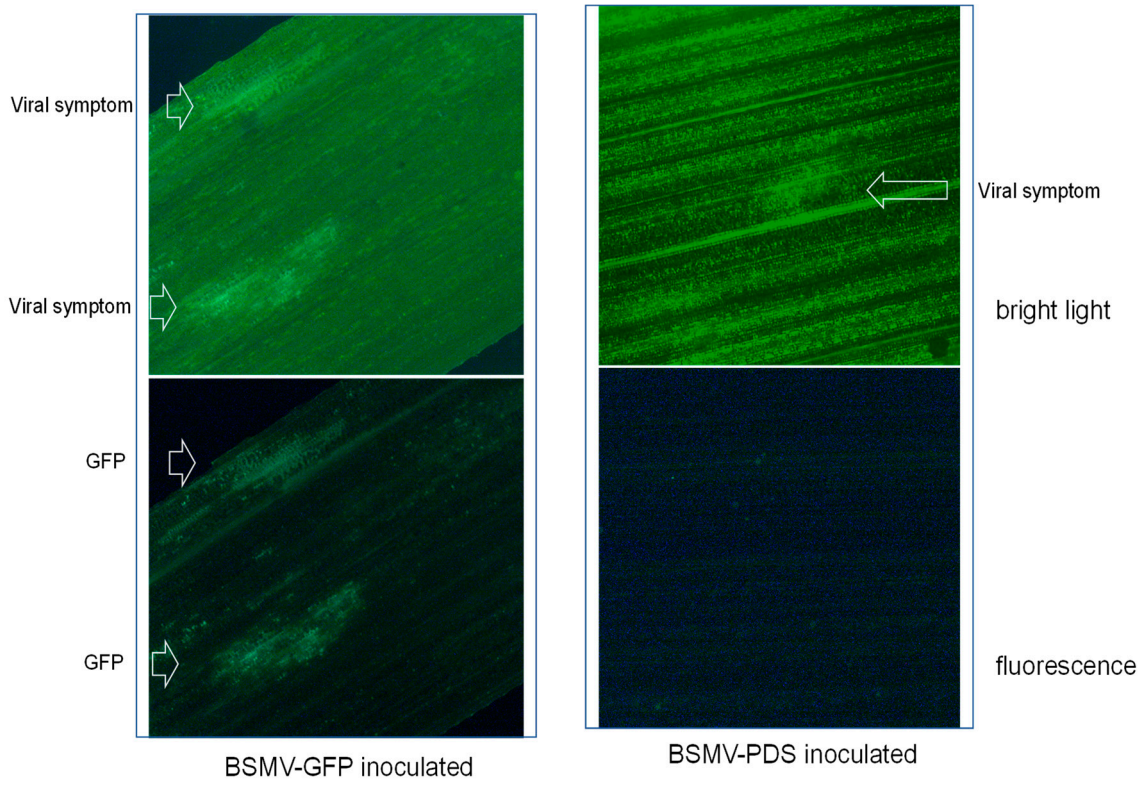

Figure 6. Microscopic images of leaf sections from BSMV-GFP and BSMV-PDS inoculated plants. Green fluorescence signals are visualized from the BSMV-GFP inoculated plants but not from the BSMV-PDS inoculated ones.

3.4. Progress and Timeline of BSMV-Mediated Assays

We inoculated BSMV by rubbing RNAs into the second leaves of *Avena* plants. We conclude that the virus started replicating in the living cells of the inoculated leaves, but in general, the viral loads were not high enough to show symptoms. Only leaves younger than the inoculated ones had viral symptoms starting at 14 dpi (Figures 2 and 7).

The principle of BSMV-mediated gene overexpression is to use the virus to introduce and increase the target gene transcripts during the infection and genome replication processes. Therefore, only cells containing virus will have the target gene transcripts and proteins, as seen by GFP signals that overlapped with viral lesions symptoms on BSMV:GFP inoculated oats (Figure 7). In contrast, BSMV-mediated gene silencing is a host anti-viral defense activated by the presence of double-stranded RNAs during BSMV replication, and only occurs after the virus enters host cells and successfully replicates to levels detectable by the host. In these studies, Avena plants showed viral symptoms on the second leaf but not on the 3rd and subsequent leaves, indicating that host anti-viral gene silencing and degradation had been activated. For the same reason, the photobleaching phenotype due to the PDS gene silencing was only detected on newly emerged leaves of plants with viral symptoms at 18 dpi. These results correspond to those seen in virus-free leaves on plants inoculated with BSMV:00 (Figure 7).

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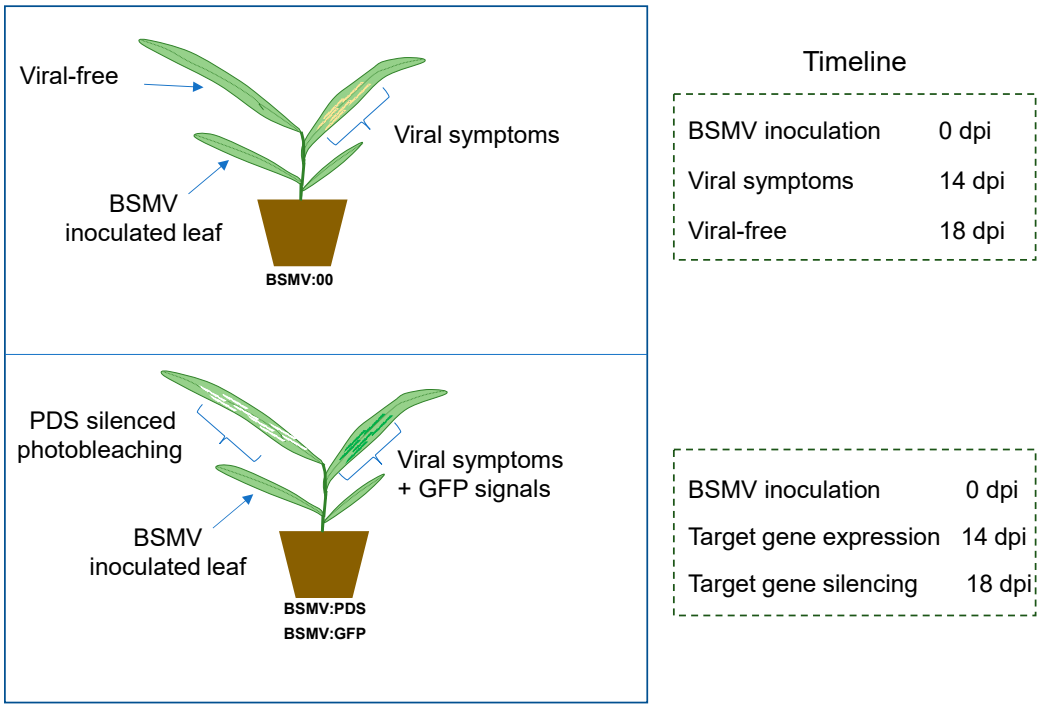

Figure 7. Progress and timelines of BSMV-mediated assays in oats.

4. Discussion

4.1. Challenges of the BSMV-Mediated Assays

Our results demonstrate that BSMV can be used to silence, knock down, or overexpress genes of interest in two *Avena* species. We conclude that assay success depends on two prerequisites: good viral inoculation and stabilization of the inserted fragment. Both aspects can result in target-gene silencing and over-expression, but only if successful virus replication occurs in live cells of the host plant. A key requirement is to balance the level of cell wounding to allow the viral RNAs to enter without excess leaf damage. Finding the appropriate pressure for rubbing inoculation takes practice, and achieving the balance of wounding the cells and recovery is relatively easier on young leaves. Therefore, we recommend inoculating at least 10 plants for each treatment at a very early growth stage, as early as the 2-leaf stage.

Loss of foreign DNA or the instability of inserted fragments can be a general problem of using virus-mediated assays (26). There is also a minimum requirement of 120 bp for insert size to achieve sufficient silencing (27,28). For BSMV, a 120- to 500-bp insert size is recommended to avoid the loss of the fragment by the virus within the first 10 replications (29,30).

Our results showed that BSMV could produce viral symptoms in oats, but the symptoms were mild and delayed as compared to wheat (Figure 2), suggesting that the wheat cellular machinery is more suitable for BSMV replication and movement than that of oats. The results also indicated to us that intermediate transcript levels of the inserted gene were produced by the virus in oats, leading to lower levels of short dsRNAs produced by the initial host silencing machinery. This issue could potentially be overcome by increasing the amount of BSMV RNAs for inoculation to increase the initial BSMV viral loads and thus achieve higher target gene transcripts.

4.2. Advantages and Disadvantages

Based on the results shown here, we conclude that levels of the inserted fragment were increased at 14 dpi, but then decreased due to dsRNA-mediated degradation at 18 dpi (Figs. 2, 3 7). A potential advantage of BSMV-mediated assays is that the same host endogenous gene could be overexpressed and silenced with one inoculation of the same plant if the full-length gene is inserted in the orientation

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shown in Figure 1B. However, the instability of genes >500 bp increases significantly, so that the virus would lose the inserted DNA within 10 replications (29,30). We conclude that BSMV-mediated gene manipulation is not an ideal choice for genes >500 bp.

Research on weed evolution, population genetics, and resistance mechanisms to one or more herbicides has been limited because of a lack of genetic resources and tools. However, this situation has improved in recent years as resources for weedy species are made available. For example, more than 30 weed genomes have now been sequenced, stable transformation systems are being developed, and one application of VIGS-mediated gene silencing has been reported (31). In our laboratories, current work focuses on the creation of recombinant inbred lines and resulting quantitative trait locus (QTL) mapping for MHR (32). Establishing VIGS- and VOX-mediated gene manipulation protocols for *A. fatua*, a serious worldwide weed, can now be included in these advances.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org. File S1: *Avena fatua* PDS sequence and region used for gene silencing; File S2: BSMV gamma vector plus GFP and RBS sequence

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