# Resources for Virus-Induced Gene Silencing in the Grasses[1]

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Virus-induced gene silencing (VIGS) is a very useful research tool for rapid creation of gene knockdown phenotypes that can be used to assess plant gene function (Kumagai et al., 1995; Ratcliff et al., 1997; Baulcombe, 1999). VIGS exploits the fact that infection by many RNA viruses activates a conserved, RNA-based plant antiviral defense response, which targets the RNA produced by infecting viruses for sequence-specific degradation (Ratcliff et al., 1997). By inserting a fragment of sequence into the viral vector from a plant gene under study, transcripts of the gene also become targets for degradation, thus causing the gene of interest to be significantly down-regulated or knocked down.

Several aspects of VIGS make it a particularly useful tool for plant functional genomics studies. First, it is a rapid experimental procedure. In most instances, the knockdown phenotype of a gene of interest can be generated within 1 to 2 months of identifying the target sequence. This is far quicker than what is possible through the production and analysis of knockout mutants or stably transformed RNAi plants (Burch-Smith et al., 2004). Second, VIGS does not require full-length cDNA sequences to function, so experiments can be initiated without complete gene sequence information. Third, silencing is initiated by infecting plants with the VIGS construct, so silencing occurs transiently and the VIGS phenotype affects only a portion of the plant. This is unlike what occurs in stable RNAi or mutant plants where the loss-of-function phenotype occurs throughout the plant, thereby increasing the occurrence of lethal phenotypes, which can limit gene function evaluations. Related to this, VIGS can be performed on species that are difficult to transform for stable RNAi studies. Fourth, VIGS can be particularly useful for research in polyploid plants because gene silencing occurs through homology-dependent RNA-mediated gene silencing, and therefore any genes sharing at least 85% sequence identity are likely to be down-regulated (Kumagai et al., 1995; Holzberg et al., 2002). In this way, knockdown phenotypes can be observed because the closely related homeologous genes present in polyploids are likely to be silenced as well.

However, one major limitation to the widespread adoption of VIGS has been the lack of suitable VIGS vectors for different plant species. Initially, VIGS was almost exclusively performed in *Nicotiana benthamiana* using vectors derived from *Tobacco mosaic virus* (Kumagai et al., 1995), *Potato virus X* (Ratcliff et al., 1997), and *Tobacco rattle virus* (Ratcliff et al., 2001; Liu et al., 2002b). In recent years, new protocols and vectors have expanded the list of dicotyledonous plants in which VIGS can be employed (e.g. tomato [*Solanum lycopersicum*; Liu et al., 2002a], Arabidopsis [*Arabidopsis thaliana*; Burch-Smith et al., 2006; Pflieger et al., 2008]), and potato [*Solanum tuberosum*; Brigneti et al., 2004; Faivre-Rampant et al., 2004]), but it was not until the report of silencing in barley (*Hordeum vulgare*) using barley stripe mosaic virus (BSMV)-based vectors that VIGS became an option for functional genomics research in monocotyledonous plants and, more specifically, the grass species (Holzberg et al., 2002). This article will describe the VIGS systems currently in use in grass species and discuss what has been learned about their capabilities and limitations as functional genomics research tools.

## VIGS SYSTEMS FOR GRASS SPECIES

### BSMV-Based VIGS

A clone of BSMV was made into a vector for use in barley (Holzberg et al., 2002) and wheat (*Triticum aestivum*; Scofield et al., 2005; Tai et al., 2005), and currently is the most widely employed grass VIGS vector (Table I). BSMV is a positive-strand RNA virus of the genus *Hordeivirus*. Its genome is tripartite, composed of the $\alpha$, $\beta$, and $\gamma$ RNAs. Three DNA plasmids, each carrying a full-length clone representing the $\alpha$, $\beta$, or $\gamma$ viral RNAs, were constructed to allow in vitro production of infectious transcripts (Petty et al., 1989).

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**Table I.** *Cloned viruses with proven or potential utility for VIGS in monocotyledonous hosts*

Abbreviations not defined in the text: CymMV, *Cymbidium mosaic virus*; MSV, *Maize streak virus*; WDV, *Wheat dwarf virus*; ComYMV, *Commelina yellow mosaic virus*; RTBV, *Rice tungro bacilliform virus*; PanSV, *Panicum streak virus*; SCBMV, *Sugarcane bacilliform virus*; BaMMV, *Barley mild mosaic virus*; BYDV, *Barley yellow dwarf virus*; BrSMV (BStMV), *Brome streak mosaic virus*; MCMV, *Maize chlorotic mottle virus*; CymRSV, *Cymbidium ringspot virus*; RYMV, *Rice yellow mottle virus*; TMV-R, *Tobacco mosaic virus*, Rakkyo strain; PMV, *Panicum mosaic virus*; ORSV, *Odontoglossum ringspot virus*; WSMV, *Wheat streak mosaic virus*; FoMV, *Foxtail mosaic virus*; SBWMV, *Soil-borne wheat mosaic virus*; MNeSV, *Maize necrotic streak virus*.

| Viruses | Type/Genus | Host[a] | Infectious Form | Adapted for VIGS[b] | Ref. |
|---|---|---|---|---|---|
| BSMV | RNA/*Hordeivirus* | *Hordeum vulgare* | cDNA | + | Hu et al. (2009); Meng et al. (2009) |
| BSMV | RNA/*Hordeivirus* | *H. vulgare* | Transcript | + | Holzberg et al. (2002); Hein et al. (2005); Bruun-Rasmussen et al. (2007); Oikawa et al. (2007) |
| | | *Triticum aestivum* | Transcript | + | Scofield et al. (2005); Tai et al. (2005) |
| BMV | RNA/*Bromovirus* | *Oryza sativa*<br>*Zea mays*<br>*H. vulgare* | Transcript | + | Ding et al. (2006) |
| | | *Festuca arundinacea* | | | This publication; X.S. Ding and R.S. Nelson (unpublished data) |
| CymMV | RNA/*Potexvirus* | *Phalaenopsis* sp. | Transcript | + | Lu et al. (2007) |
| MSV | DNA/*Mastrevirus* | *Z. mays* | cDNA | − | Grimsley et al. (1987) |
| WDV | DNA/*Mastrevirus* | *T. aestivum* | cDNA | − | Hayes et al. (1988), Woolston et al. (1988) |
| ComYMV | DNA/*Badnavirus* | *Commelina* sp. | cDNA | − | Medberry et al. (1990) |
| RTBV | DNA/*Tungrovirus* | *O. sativa* | cDNA | − | Dasgupta et al. (1991) |
| PanSV | DNA/*Mastrevirus* | *Z. mays*<br>*Panicum maximum*[c] | cDNA | − | Briddon et al. (1992) |
| SCBMV[d] | DNA/*Badnavirus* | *O. sativa*<br>*Musa acuminata* | cDNA | − | Bouhida et al. (1993) |
| BaMMV | RNA/*Bymovirus* | *H. vulgare* | cDNA | − | Meyer and Dessens (1997) |
| BYDV | RNA/*Luteovirus* | *H. vulgare* | cDNA | − | Moon et al. (2001) |
| BrSMV BStMV[e] | RNA/*Tritimovirus* | *H. vulgare*<br>*Avena sativa*<br>*Phalaris paradoxa*<br>*T. aestivum*<br>*Triticum secale* | cDNA | − | Stephan et al. (2008) |
| BYDV | RNA/*Luteovirus* | *Triticum monococcum*<br>*A. sativa* | Transcript | − | Young et al. (1991) |
| MCMV | RNA/*Machlomovirus* | *Z. mays* | Transcript | − | Scheets et al. (1993) |
| CymRSV | RNA/*Tombusvirus* | *Cymbidum* sp. | Transcript | − | Burgyan et al. (1990) |
| RYMV | RNA/*Sobemovirus* | *O. sativa* | Transcript | − | Brugidou et al. (1995) |
| TMV-R | RNA/*Tobamovirus* | *Allium chinense*[f] | Transcript | − | Chen et al. (1996) |
| PMV | RNA/*Panicovirus* | *Pennisetum glaucum* | Transcript | − | Turina et al. (1998) |
| ORSV | RNA/*Tobamovirus* | *Oncidium* Gower Ramsey | Transcript | − | Yu and Wong (1998) |
| WSMV | RNA/*Tritomovirus* | *T. aestivum*<br>*Z. mays*[c] | Transcript | − | Choi et al. (1999) |
| FoMV | RNA/*Potexvirus* | *H. vulgare* | Transcript | − | Robertson et al. (2000) |
| SBWMV | RNA/*Furovirus* | *T. aestivum* | Transcript | − | Yamamiya and Shirako (2000) |
| MNeSV | RNA/*Tombusvirus*[g] | *Z. mays* | Transcript | − | Scheets and Redinbaugh (2006) |

[a]Hosts listed are from referenced publication. Other species may be hosts and readers should review later literature for each virus. [b]If a clone exists for VIGS, none of the earlier clones yielding infectious transcript or infectious cDNA is listed. [c]Prior passage in alternate host. [d]Not tested on *Saccharum officinarum*. [e]Official acronym: International Committee on Taxonomy of Viruses. [f]Evidence of infection of this host by transcript not reported in manuscript. [g]Unofficial genus categorization.

Infection is initiated by mixing in vitro transcripts from the $\alpha$, $\beta$, and $\gamma$ DNA plasmids together and rub inoculating them onto susceptible host plants. Typically, in BSMV-VIGS studies, a 120- to 500-bp fragment, representing a portion of a transcribed sequence from a plant gene, is inserted into the $\gamma$ RNA plasmid at restriction sites immediately 3′ to the stop codon of the $\gamma$b gene (Holzberg et al., 2002). An example of silencing of wheat phytoene desaturase (PDS) expression by BSMV-mediated VIGS is shown in Figure 1A. The 120-bp minimum size for the plant gene fragment is based on the observation that host insert sequences $<$120 bp are significantly less effective in BSMV-VIGS (Scofield et al., 2005; Bruun-Rasmussen et al., 2007). The

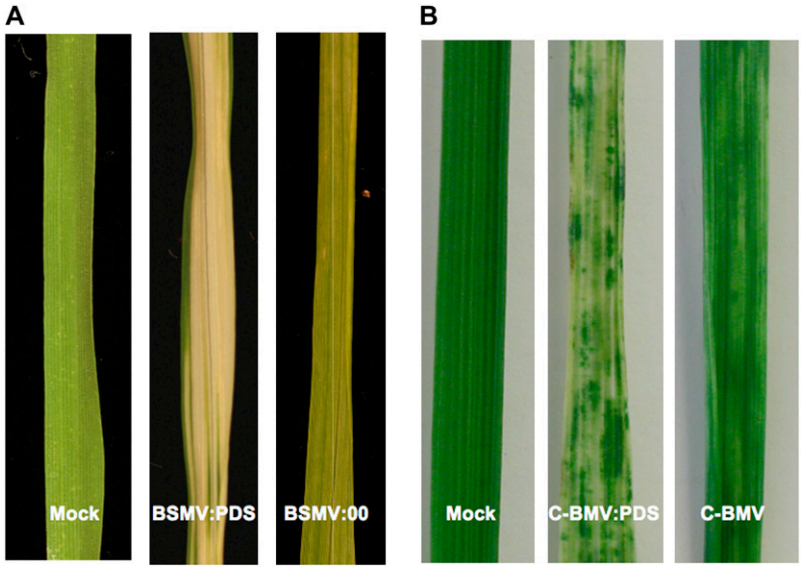


**Figure 1.** Examples of VIGS of PDS expression in wheat and tall fescue. Wheat (A) and tall fescue (B) leaves were photographed 15 d postinoculation with buffer (mock) or with the indicated BSMV or BMV transcripts. BSMV:00, BSMV with no plant sequence inserted; BSMV:PDS, BSMV carrying fragment of PDS (Scofield et al., 2005); C-BMV, chimeric BMV. Chimeric BMV is composed of RNAs 1 and 2 and modified 3 of the fescue strain of BMV (F-BMV; Ding et al., 2006). C-BMV:PDS, C-BMV carrying fragment of PDS.

upper size limit of 500 bp is less well defined, but reflects the fact that all sequences inserted into plant viral vectors are unstable as the virus replicates (Pogue et al., 2002) and larger fragments may be lost with greater frequency (Bruun-Rasmussen et al., 2007; Cakir and Scofield, 2008).

The temporal and spatial patterns of gene silencing have been analyzed for BSMV-VIGS in wheat and barley seedlings. When BSMV infection is initiated on the second leaf, BSMV moves systemically into the third leaf and significant silencing can be detected there 3 d postinoculation and will persist until at least 21 d postinoculation (Hein et al., 2005; Scofield et al., 2005). This pattern of silencing has proven sufficient to allow the initiation of VIGS and the subsequent challenge of silenced tissue with a pathogen. In this way, BSMV-VIGS has been used to demonstrate the functional requirement of particular genes for resistance in a wide range of grass disease systems, including the wheat *Lr21*-mediated leaf rust resistance pathway (Scofield et al., 2005), the wheat stripe rust resistance pathway (Zhou et al., 2007), the barley *Mla13* pathway (Hein et al., 2005), and nonhost resistance of barley to *Cochliobolus carbonum* race 1 (Sindhu et al., 2008).

Three variations of the BSMV-based VIGS vector should be mentioned to illustrate their potential benefits and weaknesses. Holzberg et al. (2002) tested a variant strategy in which the $\beta$a gene, encoding the coat protein, was deleted from the $\beta$ RNA. This deletion gave larger areas of photobleaching in VIGS experiments directed toward silencing the barley *PDS* gene. However, it was reported that the use of this coat protein deletion construct increases the severity of symptoms due to virus infection, which could interfere with the observation of some plant gene-silencing phenotypes (Scofield et al., 2005). A second variant was recently reported by the Wise laboratory, where the plasmids encoding the $\alpha$, $\beta$, and $\gamma$ RNAs were engineered to be infective as DNA plasmids when biolistically bombarded into barley, thus avoiding the expense of in vitro transcription (Hu et al., 2009; Meng et al., 2009). To accomplish this, the T7 promoter was replaced by a cauliflower mosaic virus 35S promoter and a ribozyme sequence was introduced downstream of the viral cDNA sequence for each plasmid to create the proper 3′ end after transcription in planta. Using this system, the three DNA plasmids are mixed, bombarded into barley to initiate viral infection, and then plant sap containing large amounts of infectious virus is extracted from these plants that can then be used to infect many plants for VIGS studies. A third variation of the BSMV-VIGS vector was explored by Tai et al. (2005), where a *Bam*HI restriction site for cloning plant gene fragments was engineered into the ATG codon of the $\gamma$b gene. With this cloning strategy, $\gamma$b is either not expressed or, if its translation can be initiated from an in frame ATG provided by the plant gene sequence, it is synthesized as a fusion protein with the N-terminal amino acids encoded by the plant gene insert. Because of the potential variation in translation efficiency between constructs with different inserts, the effectiveness of this vector may vary. Nonetheless, this vector silenced PDS, subunit H of magnesium-protoporphyrin chelatase complex, and $\beta$7 subunit of the 20S proteasome complex. As yet, there is no published side-by-side comparison of the silencing efficiency achieved with BSMV-VIGS vectors engineered to carry plant gene fragments inserted at the 5′ or 3′ ends of $\gamma$b.

To date, all the published research employing BSMV-VIGS has used a seedling assay. However, by inoculating upper leaves of older wheat plants with the BSMV RNAs, it was determined that VIGS can be achieved in the flag leaf and floral organs (S.R. Scofield, unpublished data). The ability to silence in the adult structures of wheat should make it possible to apply VIGS to dissect the genetic pathways controlling flowering,

seed development, grain quality, and pathogen defense in floral tissues.

### Brome Mosaic Virus-Based VIGS

More recently, a second VIGS system based on *Brome mosaic virus* (BMV) was developed for rice (*Oryza sativa*), maize (*Zea mays*), and barley (Ding et al., 2006). The genome of BMV is also tripartite and the three RNAs, designated RNAs 1, 2, and 3, have been cloned in a similar manner as for BSMV, such that infectious RNAs can be produced by in vitro transcription. The Nelson laboratory produced a hybrid (H)-BMV vector for VIGS from plasmids containing BMV RNA 1 and 2 sequences from a novel *Festuca* (F)-infecting strain of BMV (Rouf Mian et al., 2005) that infects rice and an RNA 3 sequence from a BMV that does not infect rice. RNAs 1 and 2 were determined to contain the host range determinant for F-BMV (Ding et al., 2006). Additionally, they made an effort to reduce disease symptoms caused by H-BMV during VIGS by incorporating sequences from a mutant RNA 3, known to enhance BMV replication in plants, into the RNA 3 of F-BMV. The modified virus, which was designated C-$BMV_{A/G}$, accumulated to a 2-fold higher level in cells than the parental F-BMV and caused milder mosaic symptoms than H-BMV in rice and other monocotyledonous host plants. The efficacy of using C-$BMV_{A/G}$ for VIGS was demonstrated by knocking down the *PDS* genes in maize and barley, and the actin and Rubisco activase genes in rice (Ding et al., 2006). The specific maize and rice cultivars in which C-$BMV_{A/G}$-VIGS was demonstrated to function were cv Va35 and cv IR64, cv IR8 and cv PI615210, respectively. In addition, the C-$BMV_{A/G}$ vector functions in tall fescue (*Festuca arundinacea*) to knock down PDS expression (Fig. 1B; Table I; X.S. Ding and R.S. Nelson, unpublished data).

## RESOURCES, LIMITATIONS, AND FUTURE DEVELOPMENT

Development of VIGS tools for grasses is at a very early stage. The BSMV and BMV systems described here will undoubtedly be found to be effective in other grasses as further testing is done. However, it is quite possible that other viruses will be identified with superior capabilities for VIGS in grass species. A list of cloned viruses that infect monocotyledonous hosts that could be tested for VIGS is given in Table I. An interesting possibility is *Tomato yellow leaf curl virus*, which can infect and express reporter genes in a very wide range of dicot and monocot plants. This virus has been modified to serve as a VIGS vector, but has not yet been tested for silencing in a monocot (Peretz et al., 2007).

For each potential or existing VIGS vector, there are constraints that may limit their ability to silence target genes. For example, it is well known by virologists that viral gene products interact intimately with their host's gene products and incompatibilities at this level cause many of the restrictions in host range observed for a given virus. In this regard, it is interesting to note that a single nucleotide change is known to limit BSMV's pathogenicity on oat (*Avena sativa*; Weiland and Edwards, 1996). By this line of reasoning, it is also possible that nucleotide changes will be found that can expand the range of plants in which these monocotyledonous VIGS tools can be employed. The BMV silencing vector is one such example because it utilizes genomic RNAs from a strain that infects rice, unlike the closely related, highly studied Russian strain (Ding et al., 2006).

One of the greatest difficulties with VIGS in monocotyledonous plants is the transient nature of the gene silencing. Production of useful knockdown phenotypes for functional genomics requires a reliably large area of the plant to manifest the loss-of-function phenotype so that it can be easily observed. The size of area of the plant expressing the knockdown phenotype reflects a very delicate balance between the pathogenesis and accumulation of virus and strength of the silencing response produced by the host plant. The factors controlling the extent of the development of the VIGS phenotype are not well understood; however, it is clear that the state of plant growth is important. Viral movement and spread of the silencing signal are driven by source-sink relationships (Tournier et al., 2006) and careful attention to uniform plant growth conditions is crucial for reproducible VIGS phenotypes (Burch-Smith et al., 2004; Robertson, 2004).

Another factor that may explain the transient nature of the gene-silencing phenotype in monocotyledons is the previously mentioned instability of fragments cloned into plant viruses. Sequences inserted in viral vectors may be deleted and results presented by Bruun-Rasmussen et al. (2007) indicate that insert instability is directly related to insert length and that the transient nature of VIGS over time correlates with the extent of the loss of the plant gene insert. Although it has been reported that inverted repeat sequence inserts improve silencing efficiency (Lacomme et al., 2003), it should be recognized that such constructs may be unstable in the virus vector. Besides the length or duplex-forming capacity of the insert, the sequence of the insert itself may influence stability. With the BMV vector, an insert from an actin gene was more stable than an insert from a PDS gene, although the actin insert was longer (398 bases versus 240 bases; Ding et al., 2006).

The presence and strength of viral suppressors in the virus vector or expressed as a transgene, will modify the silencing phenotype. It was recently shown that the tobacco mosaic virus 126-kD protein suppressor expressed as a transgene in *N. benthamiana* allowed a stronger silencing phenotype when expressed at a low level versus either a high level or in its absence (Harries et al., 2008). It was hypothesized that the suppressor expressed at a low level allowed a higher level of the virally encoded host gene transcript to be targeted by the silencing system. Although the presence of a suppressor in an infectious virus vector often inhibits VIGS

(e.g. for review, see Voinnet, 2005), based on the results described above where the suppressor level was modulated in trans, the possibility exists to modulate suppressor activity in cis to improve VIGS.

The successful application of VIGS as a tool in gene function studies requires that disease symptoms induced by VIGS vectors not interfere with the loss-of-function phenotype associated with silencing the target gene. This confounding result has been observed for some BSMV-barley cultivar combinations (Hein et al., 2005; Scofield et al., 2005), as well as particular rice cultivar-BMV combinations (X.S. Ding and R.S. Nelson, unpublished data). This issue may be very important in experiments studying specific gene alleles that are only available in particular cultivars (e.g. disease resistance genes). This possibility should be tested by researchers at the outset for each new plant cultivar-virus silencing study. Careful observation of plants infected with a control VIGS construct containing either no plant gene fragment or a nonplant gene fragment of the same length as inserts under study (e.g. a GFP fragment; Hein et al., 2005) is critical in addressing this issue.

For all grass species other than rice, the complete genome sequence is not available for guiding the design of VIGS inserts. This reality must always be considered when a negative result is observed in a VIGS experiment. The existence of gene family members that are sufficiently divergent at the nucleotide level to escape silencing and mask the appearance of a knockdown phenotype is theoretically possible. Additionally, the possibility of off-target silencing must be considered when a positive result is obtained in a silencing study (Xu et al., 2006). Here, the concern is that a VIGS phenotype actually results from the silencing of a gene that was not intentionally targeted for silencing, but has sufficient homology to be down-regulated by the VIGS construct. In the absence of complete genome sequence, a very effective control to rule out such a possibility is to perform additional VIGS experiments using nonoverlapping sequences from the gene of interest. If these additional constructs result in the same VIGS phenotype, the possibility of off-target silencing becomes extremely remote.

VIGS can be employed in high-throughput forward genetic screens in which random cDNA collections are screened for knockdown phenotypes (Lu et al., 2003). Neither the BSMV nor the C-BMV$_{A/G}$ constructs can be employed in such high-throughput studies as they exist now. However, they can be used for moderate throughput studies (we estimate that a team of three researchers should be able to assess more than 100 genes per year once a system is optimized). Great improvement in experimental throughput would be achieved if these VIGS systems could be engineered so that the viral infection was initiated by T-DNA transfer of constructs that then express infectious viral RNAs, rather than requiring large-scale production of in vitro transcripts, infectious plasmids, or plant sap containing infectious VIGS virus. Efforts are under way to develop such constructs for BSMV and BMV. Also, the ability to insert cDNA libraries into the VIGS vectors that infect monocotyledonous plants without using restriction enzymes is being pursued, as was accomplished for the tobacco rattle virus-based silencing vector that infects dicotyledonous plants (Dong et al., 2007).

In summary, the existing BSMV and BMV vectors can be used for VIGS studies in the main grass crops, barley, maize, rice, and wheat. VIGS studies with both vectors have been completed with reporter genes, for proof of concept, and with some genes of interest. With time, these vectors will be further enhanced to provide high-throughput rapid screening for monocotyledonous species and to provide a longer lasting and more widespread silencing phenotype. In addition, the search for other monocotyledonous plant-infecting viruses that could serve as effective VIGS vectors should continue. The future looks bright for this technology because it will do nothing but improve with time.

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