

# Chapter 2

## Rationale for Developing New Virus Vectors to Analyze Gene Function in Grasses Through Virus-Induced Gene Silencing

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### Abstract

The exploding availability of genome and EST-based sequences from grasses requires a technology that allows rapid functional analysis of the multitude of genes that these resources provide. There are several techniques available to determine a gene's function. For gene knockdown studies, silencing through RNAi is a powerful tool. Gene silencing can be accomplished through stable transformation or transient expression of a fragment of a target gene sequence. Stable transformation in rice, maize, and a few other species, although routine, remains a relatively low-throughput process. Transformation in other grass species is difficult and labor-intensive. Therefore, transient gene silencing methods including *Agrobacterium*-mediated and virus-induced gene silencing (VIGS) have great potential for researchers studying gene function in grasses. VIGS in grasses already has been used to determine the function of genes during pathogen challenge and plant development. It also can be used in moderate-throughput reverse genetics screens to determine gene function. However, the number of viruses modified to serve as silencing vectors in grasses is limited, and the silencing phenotype induced by these vectors is not optimal: the phenotype being transient and with moderate penetration throughout the tissue. Here, we review the most recent information available for VIGS in grasses and summarize the strengths and weaknesses in current virus-grass host systems. We describe ways to improve current virus vectors and the potential of other grass-infecting viruses for VIGS studies. This work is necessary because VIGS for the foreseeable future remains a higher throughput and more rapid system to evaluate gene function than stable transformation.

**Key words:** VIGS, Monocotyledons, RNAi, Plant viruses, Barley, Wheat, Maize, *Brachypodium*, *Setaria*

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### 1. Introduction

Genome and EST sequencing have produced massive amounts of sequence information in the plant genomic era. Genome sequences of many grasses are available (1–4). However, the functions of many of the genes in these sequences are unknown, and determining

their functions is a major challenge for plant biologists studying these species. Gene function can be studied either by overexpression, stable gene disruption (knockout), or silencing (knockdown) procedures (5, 6). Chemical or irradiation mutagenesis, insertional mutagenesis, RNAi, and virus-induced gene silencing (VIGS) are some of techniques used to achieve gene knockout or knockdown in both monocotyledons and dicotyledons. Chemical, irradiation, and insertion mutagenesis methods are extensively used to study the function of single genes; however, they generally do not allow the analysis of gene families (7–10). Methods exist to produce stably modified plants where gene families are silenced by targeting conserved sequence domains. However, these methods involve stable transformation which for grass species is a long, if at all achievable, process. VIGS is a rapid alternative knockdown method that allows silencing of individual genes or gene families to study their function in plant development, disease resistance, and during abiotic stress (5, 11–14). As generally occurs with stable RNAi, VIGS involves sequence-specific RNA degradation at the posttranscriptional level in plants (15), but unlike stable RNAi, results can be obtained within 2 months from target identification. Approximately 37 VIGS vectors are available for studies with dicotyledonous plants, and over 37 dicotyledonous species have been studied with these vectors. Findings from this research have resulted in a greater understanding of vegetative and reproductive plant development, biotic and abiotic stress tolerance, and nodule development (11).

For grass species, fewer VIGS vectors are available and thus fewer species (11 crop plants) have been studied (16). Although VIGS has been applied in grass species to study genes involved in biotic stress tolerance and cell wall biosynthesis (16, 17), there are some significant limitations apparent in these studies compared with those involving dicotyledonous plants (particularly those involving *Nicotiana benthamiana*). Highest among these is the loss of the silencing phenotype with time as the plant develops. In this review, we will provide information on the VIGS vectors available for functional genomic studies in grasses, some breakthrough findings using these vectors, the current limitations in VIGS studies in grasses, and ways to improve this situation both by modifying current vectors and identifying new vectors.

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## 2. VIGS Vectors Available for Grasses

In the past 9 years, three VIGS vectors have been developed for grass species: *Barley stripe mosaic virus* (BSMV; (18–20)), *Brome mosaic virus* (BMV; (21, 22)), and *Rice tungro bacilliform virus*

(RTBV; (23)). A brief description of each vector and their inoculation protocols follows:

### 2.1. BSMV-VIGS Vector

BSMV is a positive-strand RNA virus of the genus *Hordeivirus*. It has a tripartite genome composed of  $\alpha$ ,  $\beta$ , and  $\gamma$  segments. Holzberg et al. (18) modified a clone of the ND18 strain of BSMV to function as a VIGS vector by deleting the coat protein gene within the plasmid representing the  $\beta$  genomic RNA and adding a multiple-cloning site (*PacI* and *NotI*) downstream of the  $\gamma$ b gene for insertion of foreign gene fragments (120–500 bp). Bruun-Rasmussen et al. (24) created a similar BSMV vector by inserting sequence containing slightly different restriction sites (*SmaI*, *PacI*, and *BamHI*) downstream of the  $\gamma$ b gene and maintaining the coat protein gene in the  $\beta$  genomic RNA. The inclusion of the coat protein gene decreased undesirable necrosis on infected barley leaves (24, 25). Later, Meng et al. (20) inserted the three BSMV cDNAs from the Holzberg et al. (18) constructs, the  $\beta$  segment containing an active coat protein gene, each into a separate binary vector between a 35S promoter and a ribozyme/nopaline synthase 3' terminator, the ribozyme added to create a more functional 3' terminal sequence after transcription of the plasmid *in planta*. A different version of the BSMV vector was developed by modifying the  $\beta$  and  $\gamma$  RNAs of the ND18 strain (19). The start codon for the coat protein in the sequence representing  $\beta$  RNA was mutated as was the start codon of the  $\gamma$ b gene in the  $\gamma$  RNA sequence to create a *BamHI* site for insertion of the foreign gene fragment. VIGS using the BSMV vectors was demonstrated in barley (18), wheat (25), ginger (26), *Haynaldia villosa* (27), *Brachypodium distachyon* (28, 29), and oat (29).

#### 2.1.1. Inoculation Methods

Inoculum for the BSMV vector developed by Holzberg et al. (18) and Tai et al. (19) is produced by *in vitro* transcription of linearized plasmids containing the BSMV genome sequences. The transcripts are capped during the reactions and the products mixed with FES buffer prior to rub-inoculation of seedlings (18, 25). Meng et al. (20) developed a modified system using particle bombardment of binary vectors containing the BSMV genomes for introduction into barley. The biolistic-based delivery system does not require expensive *in vitro* transcription enzymes, but initial costs can be extensive if the purchase of a commercial biolistic gene gun and gold particles is required.

### 2.2. BMV-VIGS Vector

BMV is a positive-strand RNA virus of the genus *Bromovirus*. It has a tripartite genome composed of RNAs 1, 2, and 3. Ding et al. (21) cloned and modified the fescue strain of BMV (F-BMV, isolated from *Festuca arundinacea*) to function as a VIGS vector. A clone of genomic RNA 3 representing the Russian strain of BMV (R-BMV) was used with genomic clones representing RNAs 1 and

2 of F-BMV to allow easy foreign gene insertion (due to a unique *Hind*III restriction site in the R-BMV RNA 3 clone) and infectivity to rice (due to the use of clones representing F-BMV RNAs 1 and 2 (21)). Foreign gene fragments were inserted downstream of the coat protein stop codon within the cDNA representing RNA 3. The RNA 3 clone representing F-BMV later was modified by replacing a portion of the intergenic sequence between movement and coat protein genes with a corresponding fragment from the clone representing the R-BMV RNA 3. This was done to increase the accumulation of RNA 3 and the subgenomic RNA 4 from RNA 3, both containing the target host gene fragment that serves as substrate for RNA silencing, during infection (30). The BMV vector containing this chimeric RNA 3 (C-BMV RNA 3), when analyzed against parental viruses, yielded more progeny virus than F-BMV and induced fewer disease symptoms than the R-BMV in rice (21). More recently, foreign gene fragments were directionally inserted into the modified F-BMV RNA 3 clone between newly added *Nco*I and *Avr*II restriction sites immediately downstream of the coat protein sequence, thus yielding a VIGS vector predominantly composed of F-BMV sequence (31). This BMV vector was further modified by cloning sequences representing RNAs 1, 2, and 3 into a modified pCambia 1300 binary vector between a double 35S promoter and a ribozyme to create a more functional 3' terminal sequence after transcription *in planta* (31). The BMV vector used by Pacak et al. (22) for VIGS was developed from R-BMV (32) for RNA recombination studies. In this vector, the R-BMV RNA 3 clone was modified by replacing the existing sequence 3' of the coat protein open reading frame (ORF) with two restriction sites separated by a 337-nt spacer, to allow expression of inverted repeats, followed by 295 nt from the 3' end of wild-type R-BMV RNA 1. VIGS using the BMV vectors was demonstrated in rice, maize, and barley (21) and also is under study in *Setaria italica* (foxtail millet) and *Sorghum bicolor* (sorghum) (H. Ramanna, X.S. Ding and R.S. Nelson, unpublished data).

### 2.2.1. Inoculation Methods

Inoculum for the older BMV vectors developed by Ding et al. (21, 33) and Pacak et al. (22) is produced by *in vitro* transcription of linearized plasmids containing the BMV genome sequences. The transcripts are capped during the reaction, and the product is mixed with FES buffer and then rub-inoculated to seedlings (21). We determined that inoculation of the BMV vector to *N. benthamiana* first, as an intermediate host, provides a high titer of virus in extract from these plants for subsequent inoculation to the grass host (33). For the DNA-based BMV vector, an *Agrobacterium*-mediated vacuum infiltration method was developed to introduce the virus into rice (31). Because the current *Agrobacterium*-mediated vacuum infiltration method is not optimized for all grasses, we infiltrate *N. benthamiana* as an intermediate host, using a needle-less syringe,

and the sap from infected leaves of this species is rub-inoculated to the target grass plants. Using this method, VIGS has been demonstrated in several grasses (e.g., foxtail millet and sorghum) (H. Ramanna, X.S. Ding and R.S. Nelson, unpublished data). We propose that this method may be useful for a wide range of grasses. Van der Linde et al. (34) further improved VIGS studies using the BMV vector by normalizing, between treatments, the vector inoculum loads obtained from the *N. benthamiana* intermediate host that were destined for the grass host.

### 2.3. RTBV-VIGS Vector

RTBV is a double-stranded DNA virus of the genus *Pararetrovirus*. It has a genome of approximately 8 kb encoding four ORFs (I to IV). RTBV was modified to serve as a VIGS vector by assembling the viral DNA molecule as a partial dimer within the T-DNA of a binary plasmid. In addition, the RTBV promoter was replaced with the constitutively expressed maize ubiquitin promoter, and a tRNA-binding site and a multiple-cloning site, the latter for the insertion of foreign gene fragments, were added. VIGS using the RTBV vector was demonstrated in rice (23).

#### 2.3.1. Inoculation Method

The RTBV vector is introduced into the host plant through *Agrobacterium*-mediated injection (23).

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## 3. Analysis of Gene Function with Available VIGS Vectors

Currently, BSMV and BMV are the only vectors widely used to characterize gene function in grasses through VIGS. BSMV vectors have been used to study genes involved in disease resistance, such as leaf rust resistance genes *Lr21* (25) and *Lr10* (35) in wheat; stem rust resistance genes *TaRLK1*, 2, and 3 in wheat (36) and contig4211 of the *NecS1* gene (*NecS1* encodes a cation/proton-exchanging protein (HvCAX1)) in barley (37); powdery mildew resistance genes *HSP90* (38), *Blufensin1* (*Bln1*) (20), and *WRKYs 1* and 2 in barley (39) and serine and threonine protein kinase gene (*Stpk-V*) in wheat (40); necrotrophic fungal resistance gene *ToxA-binding protein1* (*ToxABPI*) in wheat (41); stripe rust fungal resistance genes *TaHsp90.2* and *TaHsp90.3* in wheat (42); and genes involved in nonhost resistance in barley (43). BSMV vectors also have been used to study genes involved in insect resistance in wheat, such as the aphid resistance genes *WRKY53* and *Pal* (44); genes involved in cell wall metabolism in barley, such as *P23k* (45) and *CesA* (17); root genes involved in phosphate acquisition, such as *IPSI*, *PHR1*, and *PHO2* (29); and a gene involved in seedling growth in wheat, *TaHsp90.1* (42).

BMV vectors have been used to study maize genes involved in interactions with the fungus, *Ustilago maydis*, such as *Tps611*, *Ecb*

(CD967190), and *Bti* (BM380261). Silencing *Ecb* and *Bti* did not significantly alter leaf colonization by the virus, whereas silencing the *Tps6/11* gene increased tissue susceptibility to *U. maydis* (34). More recently, the BMV vector was used to silence an m-type thioredoxin in maize (46). Tissue silenced for the expression of this gene was more susceptible to a systemic infection by *Sugarcane mosaic virus*, a potyvirus.

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#### 4. Limitations of VIGS in Grasses and Approaches to Overcome These Limitations

Despite the significant advances made using BSMV- and BMV-based silencing vectors in grasses, these vectors are not optimized for maximum silencing efficiency in the currently utilized grass species. Also, the usefulness of VIGS in many other grasses has not been studied (12, 16). Below, we discuss the limitations for silencing evident for existing vectors and the absence of a virus vector for other grass species. We also discuss methods to improve the silencing response and the potential of other viruses as VIGS vectors.

For BSMV- and BMV-based silencing vectors, not all cultivars or accessions within a particular host species show measurable target gene silencing (21, 24, 29). This can be due to poor infectivity, replication, or movement of the silencing vector within that host. For barley, the “Black Hulless” cultivar displayed the best silencing phenotype (18, 24). In a separate study using other cultivars, “Spire” and “Clansman” exhibited the best silencing phenotype and reduction in target transcript levels (38). For wheat, the “Bobwhite” cultivar displayed more photobleaching and reduction in target transcript level than “Clark” at 22°C (47). Others saw similar results with “Bobwhite” and six undisclosed hexaploid wheat cultivars (25). None of the surveys were fully comprehensive, so other varieties may exist that provide better silencing. In a significant later study, it was determined that a BSMV vector expressing a fragment of the GFP gene (BSMV-GFP) induced expression of pathogenesis-related and phenylalanine ammonia-lyase genes, all associated with plant defense, in the wheat cultivar Renan (48). These researchers determined that the induction of these genes was correlated with enhanced resistance to *Magnaporthe oryzae*, the blast pathogen, but had no effect on the development of powdery mildew disease induced by *Blumeria graminis*. These findings make it clear that researchers must run appropriate controls to determine the influence of a virus infection itself on host metabolism irrespective of any targeted effects during VIGS.

In a survey of rice varieties, BMV caused modest mosaic symptoms on cultivars IR64, Drew, and Cypress and thus the greatest potential for minimum confounding effects on silencing studies (21). However, no BMV strain was able to infect Nipponbare, one



of the most valuable rice cultivars for plant molecular biologists and geneticists (21). Also, BMV induced severe disease symptoms (local necrosis on leaf lamina) on cultivars Moroberekan and IAC165 which would seriously confound the interpretation of findings from silencing studies. Even more serious systemic necrosis systems are induced by BMV on most cultivars of maize, including widely used B73 and Mo17 ((21), X.S. Ding and R.S. Nelson, unpublished data). These cultivars and all others that display systemic necrosis are not suitable hosts for VIGS studies. We include a table listing varieties within grass species and their responses to viruses and viral vectors used in VIGS studies (Table 1).

Although there has been considerable exploration of silencing responses within varieties for their response to the BSMV and BMV silencing vectors, the search among species for silencing responses is less complete. BMV can infect numerous species of *Poaceae* under the greenhouse conditions (49, 50), and further experiments are needed to determine if VIGS can be applied to them, particularly those recalcitrant to stable genetic transformation. One example is the use of the BMV vector to silence genes in *S. italica* (foxtail millet), a model C4 grass closely related to the prospective biofuel crop grass, *Panicum virgatum* (switchgrass) (H. Ramanna, X.S. Ding and R.S. Nelson, unpublished results). BSMV has recently been shown to be suitable for VIGS studies in *B. distachyon*, a model plant for C3 crops such as barley, wheat, and oats (29). However, it is clear from the current studies that expanding the use of the current vectors into a new grass species always will require a survey of varieties and accessions within the new target species to obtain the best silencing phenotypes.

Temperature also influences VIGS phenotypes in plants. In dicotyledonous plants, it has been shown that low temperature enhances the appearance of visible phenotypes during VIGS and higher temperatures enhance the silencing of the virus genome (5, 51–55). It is possible that the lower temperature allows continuous accumulation of the silencing substrate (i.e., the virus vector) to levels which induce gene silencing while higher temperatures lead to destruction of the substrate and no endogenous gene silencing. A similar finding was made with the BSMV vector in wheat where temperatures of 18–22°C yielded better silencing phenotypes and greater target gene transcript reductions than observed at 26°C (47). Also in barley, temperatures of 20–24°C provided better results than 16 or 28°C (24). However, for BMV, Ding et al. (56) reported that under a low temperature condition (24/20°C, day/night), this virus infected and accumulated predominantly in cells near and within vascular cells of barley. Although this study did not involve VIGS, it is important to note that even under conditions that improve gene silencing, the virus spread in the infected plants may be affected leading to an incomplete penetration of the silencing phenotype. Thus,

**Table 1**  
**Comparison of species and varietal responses during virus challenge and VIGS**

Virus	Plant species	Cultivar, variety, or PI	Response to virus	Response during VIGS	References
BSMV	<i>Hordeum vulgare</i> (barley)	Black Hullless	— <sup>a</sup>	Best	(18, 24)
		Golden Promise, Pallas, Ingrid, Chess, Simba, Relief	—	Weak, delayed silencing phenotype and viral symptom	
		Spire, Clansman, Tyne	—	Best	(38)
		Digger	—	Weak silencing and strong virus symptoms in systemic leaves	
		Clansman ( <i>Mla13</i> ), C.I. 16151 ( <i>Mla6</i> )	—	Best	(20)
		C.I. 16137 ( <i>Mla1</i> ), C.I.16155 ( <i>Mla13</i> ), Sultan-5 ( <i>Mla12</i> ), Golden promise, C.I. 16147 ( <i>Mla7</i> ), C.I.16149 ( <i>Mla10</i> ), HOR11358 ( <i>Mla9</i> ), C.I. 16143 ( <i>Mla</i> ), C.I. 15229 (Step toe), Ingrid ( <i>Mlo</i> ), Harrington, C.I. 16139 ( <i>Mlg</i> ), OWB rec, C.I. 16145 ( <i>Mlp</i> ), C.I. 16141 ( <i>Mlb</i> ), <i>mlo-5</i> BC <sub>7</sub> Ingrid, C.I. 15773 (Morex)	—	Weak silencing phenotype	
BSMV	<i>Triticum aestivum</i> (wheat)	Bobwhite	—	Best	(47)
		Clark	—	Weak silencing phenotype	
		Bobwhite	—	Best compared with six other undisclosed tested varieties	(25)



BSMV	<i>Avena sativa</i> (oat)	Belinda	Weak virus infection (compared to barley) Weak virus infection (compared to barley)	Weak silencing (compared to barley) Weak silencing (compared to barley)	(29)
	<i>Avena strigosa</i> (oat)	S75			
BMV	<i>Oryza sativa</i> (rice)	Drew, IR8, IR64, Cypress, Pokkali, M-202	Mild virus symptom	Only analyzed for IR8 and IR64: Visible, transient silencing	[(21); Ding et al. unpublished data]
		Moroberekan, IAC65 Nipponbare	Systemic necrosis No infection	–	
BMV	<i>Zea mays</i> (maize)	Va35 B73, Mo17, W22	Mild virus symptom Severe systemic necrosis	Visible, transient silencing	[(21); Ding et al. unpublished data]
BMV	<i>Setaria italica</i> (Foxtail millet)	Yugu, German R, PI 212626 PI 315088 PI 391643	Yugu and German R only, tested in detail: mild virus symptom	Best Weak silencing phenotype No silencing phenotype	Ramanna et al. unpublished data
BMV	<i>Sorghum bicolor</i> (sorghum)	BTx623 (PI564163) RioS, PI 651495, PI 651497	Mild virus symptom	Best Weak silencing phenotype	Ramanna et al. unpublished data
RTBV	<i>Oryza sativa</i> (rice)	TN-1	No stunting or yellowing	Mild, longer lasting but still transient	(23)

<sup>a</sup>–, not determined

temperature effects should always be analyzed when a new plant–virus–vector combination is chosen for studies.

Another factor that may influence silencing phenotypes during VIGS is the orientation of the foreign gene insert. Host gene fragments inserted in the sense orientation within the virus vector are never superior and in some instances are inferior to those inserted in the antisense orientation ((18, 22, 24, 25, 57) Fig. 1). Early studies indicated that use of an inverted repeat target sequence in the BSMV vector increased the visual silencing phenotype, if not the downregulation of the target sequence, compared with an antisense fragment (57). Recent studies however determined that short inverted repeats can be very unstable and less efficient than sense constructs for VIGS in BSMV and BMV (22, 29). Further work is necessary to determine the general usefulness of inverted repeats during silencing in grasses.

As hinted at in the sentence above, foreign gene fragment insert stability in the silencing vector is an important consideration during VIGS studies. Indeed, it is likely the most important factor that limits the effective use of VIGS in grasses and probably in most plant species. Researchers who utilize *Tobacco rattle virus* (TRV) to silence genes in *N. benthamiana* are among the few who can depend on the silencing phenotype to persist in new systemically infected tissue and penetrate all cell types within the involved tissue (58). This phenotype often is associated with the maintenance of the foreign gene fragment in the vector. Similar results were also obtained in soybean plants silenced for their PDS gene using the *Apple latent spherical virus*-based silencing vector (59). VIGS in grasses, however, is always associated with a transient phenotype in systemically infected tissue, usually involving only two to three leaves and the intervening stem tissues (18, 21, 24, 25). In addition, penetration of the visible silencing phenotype in leaves was often incomplete, appearing only as large or narrow stripes between longitudinal vascular bundles. These transient and incomplete visible silencing phenotypes are generally associated with the loss of the foreign gene fragment from the recombinant virus vectors during infection. Bruun-Rasmussen and colleagues studied this carefully and determined that although a larger insert (584 nt of PDS gene in BSMV) induced clear photobleaching in the second leaf above the inoculated leaf, the areal coverage was less than for those plants inoculated with BSMV containing 400 and 275 nt inserts (24). This loss of areal coverage was closely correlated with the loss of the 584 nt insert from the viral genome.

The mechanism driving the loss of a foreign gene fragment from a virus vector is not fully understood. Possible causes include deletion of the insert due to RNA polymerase hopping or recombination between viral RNAs (60–64). A recombined virus, now without an insert, will likely accumulate to very high levels compared with virus still containing an insert. Thus, loss of insert not

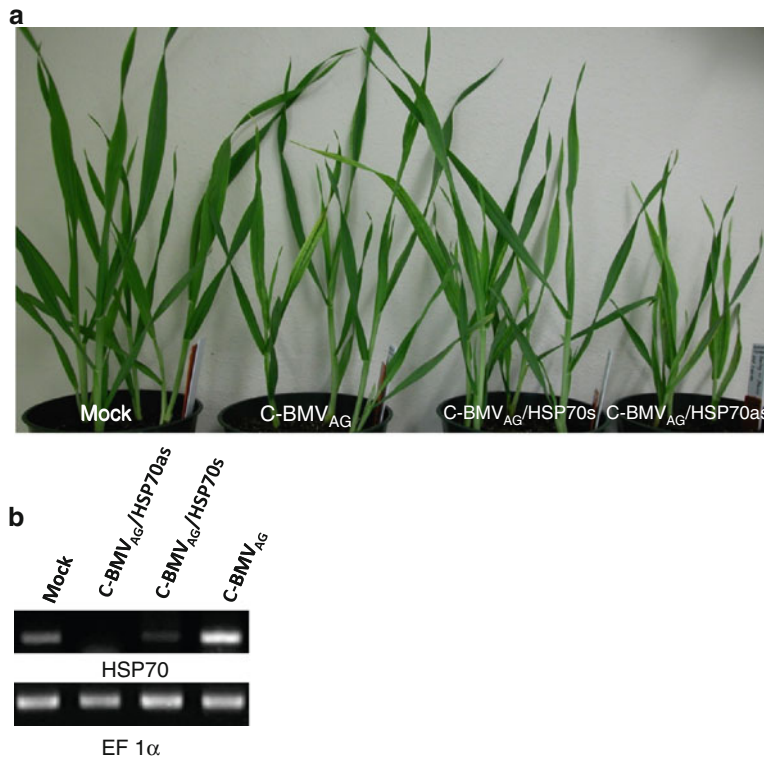


Fig. 1. Virus-induced gene silencing of a heat shock protein 70 (HSP70) was more effective with an antisense than a sense fragment insert in barley using the C-BMV<sub>AG</sub> vector. Seedlings of barley cv. Morex were inoculated with C-BMV<sub>AG</sub> or C-BMV<sub>AG</sub> carrying a sense (s) or antisense (as) HPS70 insert. The inoculated plants were grown inside a growth chamber set at 24/20°C (day/night) and photographed 10 days postinoculation (a). The second systemically infected leaves of these plants were harvested and extracted to determine the level of HSP70 transcript through semiquantitative RT-PCR (b). The barley elongation factor 1 $\alpha$  (EF 1 $\alpha$ ) gene was used as the internal control. Gel images show PCR products obtained after 30 reaction cycles. (1) Mock, (2) C-BMV<sub>AG</sub>/HSP70as, (3) C-BMV<sub>AG</sub>/HSP70s, and (4) C-BMV<sub>AG</sub>. Note the more severe stunting and greater target transcript knockdown in plants inoculated with C-BMV<sub>AG</sub>/HSP70as.

only removes the fragment responsible for inducing target gene silencing, but yields a virus that will outcompete any remaining virus that retains the insert and possibly lead to confounding virus symptoms. Bruun-Rasmussen et al. (24) observed that the BSMV vector that originally contained a 584 nt insert accumulated to much higher levels than those with 400 and 275 nt inserts, but this higher accumulation was associated with loss of the insert, in agreement with the above assertion. In a later study, this laboratory also determined that a BSMV vector with a 493 nt insert from a different gene was more stable and this was associated with a silencing phenotype (29). Thus, it is not only the length of the insert, but the nucleotide content that influences insert stability.

Virus inoculation procedures often have a significant effect on VIGS studies. In the last few years, an *Agrobacterium*-mediated infiltration procedure to introduce VIGS vectors has emerged as a rapid and reliable tool for gene silencing in *N. benthamiana*

(5, 65–68). Recent improvements of this infiltration procedure allowed researchers to achieve reliable silencing results in more plant species of the *Solanaceae* family, lettuce, *Arabidopsis thaliana* (L.), and also in fruits of tomato (69–72). In grasses, several DNA viruses were previously reported to be successfully introduced into rice, maize, and wheat through an *Agrobacterium*-mediated injection procedure (73–77). In 2009, a first report on an *Agrobacterium*-mediated vacuum infiltration procedure for VIGS in rice appeared (31). These researchers modified the BMV silencing vector by placing the viral sequences behind a doubled 35S promoter within a modified binary vector. Vacuum infiltration of rice cultivar IR64 with a solution of *Agrobacterium* C58C1 harboring plasmids containing the recombinant BMV vectors yielded much stronger and persistent silencing than those induced by mechanical inoculation of the plant with recombinant BMV vector from an intermediate host or viral RNA transcripts produced as described previously (21). This system allows inoculation of virus vector without the need for expensive in vitro transcription from plasmids. In addition, further modification of this vacuum infiltration procedure may allow researchers to conduct VIGS studies in other grass species because of the efficiency of infection. For BSMV, a recombinant vector also has been constructed using a 35S promoter and inoculated using particle bombardment, again avoiding costly in vitro transcription (20). In this instance, the three BSMV genomic sequences reside in three plasmids, while for BMV, the three genomic sequences reside in two plasmids. The BSMV system was utilized to show that silencing of BLUFENSIN1 enhanced plant resistance in compatible interactions involving the causal agent of powdery mildew disease in barley (20).

We have further improved the accumulation of the DNA-based BMV silencing vector by adding the P19 gene sequence from *Tomato bushy stunt virus* (TBSV) into the plasmid encoding the viral vector. The P19 protein of TBSV is a strong suppressor of RNA silencing (78–81). Independent expression of P19 and BMV RNA 3 from the same binary vector resulted in a higher accumulation of the virus in *Agrobacterium*-infiltrated leaves, resulting in more efficient systemic infection and RNA silencing in plants (X.S. Ding and R.S. Nelson, unpublished data). The concept of utilizing a silencing suppressor to increase virus vector accumulation and associated gene silencing has been successfully utilized through stable expression of the *Tobacco mosaic virus* 126 kDa silencing suppressor protein in *Nicotiana* sp. and challenge of these plants with a TRV silencing vector (82).

Many RNA and DNA viruses have been identified and cloned from naturally infected monocotyledonous hosts (16), and some of them have been modified as transient expression vectors to express foreign genes in leaves of monocotyledons (66, 83–85). It is likely that more RNA and DNA viruses can be found that are

suitable VIGS vectors for a wide range of monocotyledonous species. Any potential viral silencing vector should not induce severe disease symptoms in its host. It is also worthwhile to identify new viral vectors that are not transmitted by insect vectors and seed so that studies can be conducted under less stringent governmental regulations. For example, an infectious clone of *Panicum mosaic virus* (PMV) has been constructed (X.S. Ding and R.S. Nelson, unpublished). PMV is a single-stranded RNA virus (genus *Panicovirus*) and causes mild mosaic symptoms in many monocotyledonous species including *S. italica* and maize cultivar Oh28 (86, 87). PMV is readily transmitted among plants through mechanical inoculation but not through insect vectors and seed (86, 88). Modifications have been made to the PMV infectious clone to allow foreign gene fragment expression, and the usefulness of this virus to serve as a silencing vector is under investigation (X.S. Ding and R.S. Nelson, unpublished). Recently, a series of *Wheat streak mosaic virus*-based vectors were constructed by Tatineni and coworkers (85). Using these vectors, they were able to express the GFP gene in multiple grass species including barley, maize, and wheat (85). Because this virus encodes an RNA silencing suppressor (i.e., P1 protein: (89)), its application as a VIGS vector for grasses requires careful evaluation. This is also true for any “new” virus vector that is under construction. *Foxtail mosaic virus* (FoMV) is a member of genus *Potexvirus* and can cause infection in many species of Gramineae (90). This virus also has been modified and used as an expressing vector in several monocotyledons (91). Because both coat protein and the triple gene block ORFs were removed from the viral genome, the mutant virus expressed from this vector is defective in cell-to-cell and long-distance movement in its host plant. Consequently, this vector can only be used to express foreign genes or gene fragments in individual cells. Further modifications are needed prior to use of this vector for gene silencing in any host plant.

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## 5. Conclusions

VIGS in grasses is a functioning system that already has yielded significant findings in gene function studies (17, 20, 25, 34, 46). However, it is not optimized in these hosts. The existing BSMV and BMV vectors are now modified for ligation-free cloning or expression behind a 35S promoter to reduce time and cost of the procedure (20, 22, 31), but no single vector has been modified for both ligation-free cloning and expression behind a plant-active promoter. These modifications are also necessary to allow higher throughput forward and reverse genetic screens. In regard to environmental considerations, when growing the plants after

inoculation, there is a general consensus that lower temperatures provide better silencing phenotypes and target gene silencing (24, 47). Orientation of the gene insert also is an important consideration with the antisense orientation being the apparent no-cost optimum ((25, 57) Fig. 1).

Maintaining the plant gene insert in the virus vector appears to be the most important factor to control in order to obtain good silencing, being very closely correlated with the appearance of small RNAs, silencing of target mRNA, and maintenance of a visual silencing phenotype (24, 47). It is clear that insert stability is influenced both by fragment size and nucleotide constitution of the insert (24, 57). Lack of insert stability due to insert size likely explains the poor silencing obtained when two genes were targeted per insert as opposed to one (47). Maintenance of the insert may be influenced by both host and viral factors and certainly could explain variations between hosts in silencing efficiency. Although variation between host cultivars has been explored to identify those giving the best phenotype, modifications of viruses to enhance accumulation and insert stability are only in the early stages of study. Accumulation of virus vector has been enhanced by passage through a high-titer intermediate host (33, 34). Expressing a silencing suppressor at low levels in transgenic *N. benthamiana* also has been shown to improve silencing phenotypes, likely due to increasing the virus vector levels (82). Using this plant line as the intermediate host should further improve findings for grasses during VIGS by increasing the inoculum titer of the virus vector for the grass host. To improve insert stability, analysis of literature describing the structure of the viral RNA and *cis*- and *trans*-acting viral factors that influence virus accumulation and recombination rates is essential. Finding new viruses that maintain inserts better than current vectors do is certainly one way to improve the system for grasses. However, modifying existing and any new virus vectors at the molecular level to maintain stability will lead to the greatest advances toward optimizing this already powerful procedure for gene function studies.

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*Note added in proof:* A BSMV vector modified for both ligation-free cloning and expression behind a plant-active promoter was recently published: Yuan et al. 2011, PLoS ONE 6, e26468.

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