A *Foxtail mosaic virus* Vector for Virus-Induced Gene Silencing in Maize

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**AUTHOR CONTRIBUTIONS**

Y.M., C.Z., J.H.H., and S.A.W. conceived and designed the research plan; Y.M., B.K., C.Z. performed the experiments; Y.M., B.K., C.Z., J.H.H., S.A.W. analyzed the data; Y.M., B.K., C.Z., J.H.H., and S.A.W. wrote the manuscript

**ONE SENTENCE SUMMARY**

The development of an infectious clone of *Foxtail mosaic virus* and demonstration of its use for virus-induced gene silencing applications in maize is described.
Plant viruses have been widely used as vectors for foreign gene expression and virus-induced gene silencing (VIGS). A limited number of viruses have been developed into viral vectors for the purposes of gene expression or VIGS in monocotyledonous plants, and among these, the tripartite viruses *Brome mosaic virus* and *Cucumber mosaic virus* have been shown to induce VIGS in maize. We describe here a new DNA-based VIGS system derived from *Foxtail mosaic virus* (FoMV), a monopartite virus that is able to establish systemic infection and silencing of endogenous maize genes homologous to gene fragments inserted into the FoMV genome. To demonstrate VIGS applications of this FoMV vector system, four genes, *phytoene desaturase* (*pds*, functions in carotenoid biosynthesis), *lesion mimic 22* (*les22*, encodes a key enzyme of the porphyrin pathway), *iojap* (*ij*, functions in plastid development), and *brown midrib 3* (*bm3*, caffeic acid O-methyltransferase) were silenced and characterized in the sweet corn line Golden x Bantam. Further, we demonstrate that the FoMV infectious clone establishes systemic infection in maize inbred lines, sorghum, and green foxtail, indicating the potential wide applications of this viral vector system for functional genomics studies in maize and other monocots.
INTRODUCTION

Plant viruses have been widely used as vectors for foreign gene expression and virus-induced gene silencing (VIGS) (Caplan and Dinesh-Kumar, 2006; Purkayastha and Dasgupta, 2009; Senthil-Kumar and Mysore, 2011; Becker, 2015). Viral vectors capable of expressing heterologous proteins in plants and silencing endogenous plant genes provide valuable biotechnological tools to complement genetic and transgenic technologies. Plant viral vectors have many unique advantages over these other technologies including speed and ability to silence or overexpress genes in different genetic backgrounds. VIGS can knock down expression of a single gene, a gene family, or a combination of distinct genes. Due to these advantages, viral vectors have been developed and used in dicot and monocot plants.

Six different viruses have been developed into viral vectors for VIGS applications in monocots to date: Barley stripe mosaic virus (BSMV) (Scofield et al., 2005), Brome mosaic virus (BMV) (Ding et al., 2006), Cymbidium mosaic virus (CymMV) (Lu et al., 2007), Rice tungro bacilliform virus (RTBV) (Purkayastha et al., 2010), Bamboo mosaic virus (BaMV) together with its associated satellite RNA (Liou et al., 2014), and most recently Cucumber mosaic virus (CMV) (Wang et al., 2016). Among these, the BMV and CMV vectors have the ability to infect and induce gene silencing in maize (Zea mays L.) (Ding et al. 2006; Lee et al., 2015; Wang et al., 2016). There have been relatively few studies published on the application of BMV VIGS in maize (Ding et al., 2006; van der Linde et al., 2011; van der Linde and Doehlemann, 2013) suggesting that it has not been widely adopted for maize gene function analyses. The CMV vector was just recently published, and so there are not additional studies regarding its functional genomics applications in maize.

The ability of Foxtail mosaic virus (FoMV) to infect maize and other monocots makes it a candidate for viral vector development (Paulsen and Niblett, 1977). FoMV is a member of the genus Potexvirus, which is a large group of flexuous and filamentous plant viruses with a single-stranded, positive-sense genomic RNA. The type member of the potexviruses, Potato virus X (PVX), has been intensively studied as a model for this genus. The 6.435 kb genome of PVX
contains five open reading frames (ORF) (Huisman et al., 1988). ORF1 encodes the RNA
dependent RNA polymerase (RdRp), which is necessary for viral RNA replication and
subgenomic messenger RNA (sgRNA) synthesis (Draghici et al., 2009). The overlapping ORFs 2, 3,
and 4 are known as the triple gene block (TGB), and they are expressed from sgRNA1 and
sgRNA2 (Verchot et al., 1998). The TGB proteins have functions that are critical for virus
movement and suppression of host defense (Verchot-Lubicz, 2005). The final ORF, ORF5, is
expressed from sgRNA3 and encodes the only structural protein, the coat protein (CP), that is
indispensable for virus assembly and cell-to-cell movement (Cruz et al., 1998). Importantly, viral
vectors based on PVX have been developed for both gene expression and VIGS purposes, and
they have facilitated functional genomics research in PVX host plants (Sablowski et al., 1995;
Lacomme and Chapman, 2008; Dickmeis et al., 2014; Wang et al., 2014).

The full length genomic sequence of FoMV was first reported in 1991 (Bancroft et al., 1991).
Later, an RNA-based full-length infectious clone was constructed and a revised genome was
published (Robertson et al., 2001; Bruun-Rasmussen et al., 2008). Similar to PVX, the FoMV
genome contains five ORFs that include ORF1 expressed from the genomic RNA; ORFs 2, 3, and
4 expressed from sgRNA1 and sgRNA2; and ORF5 expressed from sgRNA3. One significant
difference in the genome organization of the two viruses is that FoMV has a unique ORF5A that
initiates 143 nucleotides upstream of the CP. The 5A protein is produced \textit{in vivo}, but it is not
required for replication or for systemic infection of plants (Robertson et al., 2000). Previously,
the FoMV infectious clone has been adapted as a transient gene expression vector by
substitution of the TGB or CP gene with target genes (Liu and Kearney, 2010). High levels of
foreign gene expression are obtained following Agrobacterium co-infiltration of leaf tissues
with recombinant FoMV clones plus a strong RNA silencing suppressor. This FoMV vector
system cannot systemically infect plants, because it lacks necessary movement functions
encoded by the TGB and CP.

Here, we aimed to develop a FoMV vector system that is capable of establishing whole
plant systemic infection and carrying foreign gene insertions homologous to endogenous host
genes targeted for RNA silencing. We report a new DNA-based, full-length FoMV vector system for VIGS in maize and other monocots, and we demonstrate the VIGS applications of this system by successfully silencing endogenous genes in leaves of the sweet corn variety Golden x Bantam and in the dent corn inbred line B73. Potential uses of this viral vector for functional genomics applications in maize and other monocots are discussed.

RESULTS

Construction of Infectious FoMV cDNA Clones

The full-length genomic cDNA of FoMV was obtained by two-step overlapping PCR, and it was inserted into the pSMV-NVEC plasmid at the Stul restriction enzyme cloning site (Wang et al., 2006). This construction placed transcription of the FoMV genome under control of the Cauliflower mosaic virus 35S promoter (P35S) and the nopaline synthase terminator (Fig. 1). Clones that had the correct insert size and orientation were biolistically inoculated onto sweet corn (Golden x Bantam) to test their infectivity. One clone, designated pFoMV-IA, reproducibly infected sweet corn, and so we sequenced this clone to obtain its complete genomic sequence. Analysis of the 6.183 kb FoMV-IA cDNA sequence showed that it has the expected genome organization based on comparison to previously published sequences (Fig. 1) (Bruun-Rasmussen et al., 2008). FoMV-IA-infected leaves display a mosaic pattern of light and dark green tissue, which is in contrast to the leaves of healthy non-inoculated and mock-inoculated plants (Fig. 2A). To further confirm FoMV infection, RT-PCR analysis was performed using primers designed to amplify a 295 bp fragment from the FoMV genomic RNA. The PCR product was detected in symptomatic sweet corn plants that were biolistically inoculated with pFoMV-IA, but not in non-inoculated and mock-inoculated control plants (Fig. 2B).

To enable insertion of foreign sequences into the FoMV genome, the XbaI and Xhol restriction enzyme sites were inserted immediately after the stop codon of the capsid protein (Fig. 1), and this clone was designated as pFoMV-V. Inoculation of sweet corn with pFoMV-V showed that it was infectious and induced mild mosaic symptoms that were indistinguishable
from plants inoculated with the parental pFoMV-IA clone (Fig. 2A). The symptoms were first visible within about one week after biolistic inoculation, and continued to develop through two weeks after inoculation. Under our growth conditions, sweet corn plants appear to recover from infection, because symptoms are typically observed on only the 3rd to 6th leaves. However, viral fragments were detected by PCR throughout the infected plants indicating that FoMV continues to replicate and move systemically even though symptoms decrease (Fig. 2B). From here on, names of viral constructs will be preceded with a “p” only when we are referring specifically to the plasmid DNA construct.

**Silencing of the Maize phytoene desaturase (pds) Gene Using the FoMV Vector**

To test the ability of FoMV to induce silencing of an endogenous maize gene, we first tested *pds* because it provides a striking visual marker when its expression is reduced sufficiently to cause a photo-bleaching phenotype (Holzberg et al., 2002; Ding et al., 2006). A 313-bp fragment corresponding to the 3’ end of the *pds* ORF was inserted in the antisense orientation at the *Xba*I and *Xho*I cloning site of pFoMV-V. Sweet corn seedlings at the two-leaf stage were inoculated with the pFoMV-PDS construct by biolistic bombardment. Photo-bleaching was first observed at about ten days after inoculation with pFoMV-PDS, and this phenotype became more obvious at two weeks after inoculation (Fig. 3A). The phenotypes caused by FoMV-PDS were observed from the 3rd to the 10th leaf, with the majority of plants displaying photo-bleaching from the 4th to the 9th leaf. Among the several replications of this experiment, from 10-71% of inoculated plants became systemically infected with FoMV-PDS.

To evaluate the effectiveness of VIGS of the target gene, *pds* mRNA transcript levels were compared among control plants (mock treated or infected with the empty FoMV-V) and FoMV-PDS-infected sweet corn leaves with photo-bleaching phenotypes. Total RNA was extracted from the 4th leaves of these plants, and the accumulation of *pds* mRNA transcripts was quantified using qRT-PCR. The expression level of *pds* was similar between FoMV-V infected and non-inoculated sweet corn leaves demonstrating that FoMV infection alone did not significantly affect *pds* mRNA expression (Fig. 4). In contrast, FoMV-PDS infection resulted in a significant reduction of *pds* expression in photo-bleached leaves, with transcript levels reduced
to 13.5%-27.6% of the non-silenced controls. In addition, *pds* transcript levels were quantified using qRT-PCR in leaves of plants that had been inoculated with pFoMV-PDS but were asymptomatic and did not display the photo-bleaching phenotype. As expected, we observed that *pds* mRNA levels were not significantly reduced (Fig. 4; samples AS1, AS2, AS3). These results show that asymptomatic leaves from plants inoculated with the silencing construct contain similar expression levels to those of the mock and empty vector controls, and they further confirm that reduced levels of *pds* mRNA transcripts are correlated with the photo-bleaching phenotype.

To test whether the silencing effect of FoMV-PDS could be passaged, the sap from leaves of the biolistically-inoculated plants displaying obvious photo-bleaching was used to rub-inoculate the first two leaves of naïve sweet corn seedlings. Photo-bleaching occurred after eight to nine days on the rub-inoculated plants demonstrating that the ability to induce the *pds* silencing phenotype could be passaged (Fig. 3B). Similar to procedures described above, *pds* mRNA expression levels were evaluated for leaves four and five (L4 and L5) on rub-inoculated plants displaying photo-bleaching on two independent rub-inoculated plants. We observed that the expression of *pds* was significantly reduced in the photo-bleached leaves of the rub-inoculated plants (Fig. 4). Taken together, these data showed that *pds* was silenced to a similar extent when leaf sap from pFoMV-PDS-inoculated plants was rub-inoculated onto new sweet corn plants.

**Stability of the 313 bp *pds* Fragment in FoMV-PDS**

We observed that most of the FoMV-PDS-infected plants had photo-bleaching on the 4th to 9th leaves. However, the phenotype was not uniform across all leaves and could be described as a gradient from bottom to top. The most clear and obvious photo-bleaching was observed on the 5th and 6th leaves, and then the phenotype became less severe in the upper leaves. We reasoned that this lack of uniform phenotype could be due to instability of the 313 bp *pds* fragment. To investigate the stability of the *pds* fragment in FoMV-PDS and its relationship to *pds* silencing throughout maize plants, we conducted RT-PCR analyses on RNA extracted from the following leaves: 4, 6, 9 and the top-most leaf (usually 13, but occasionally 12 or 14). These
leaves are designated as L4, L6, L9, and L top. Each leaf was collected after the leaf above it had 
emerged from the whorl, except for the top leaf, which was collected right after the tassel 
became visible (i.e. we did not collect all the leaves at the same time off a single plant at the 
end of the experiment). The RT-PCR assays used FoMV primers that flanked the cloning site to 
detect intact FoMV-PDS or deletion derivatives, and qRT-PCR was used to quantify pds mRNA 
accumulation in the same samples (Fig. 5, graphs show relative pds mRNA expression and the 
gel images show corresponding FoMV-PDS PCR products). A total of 12 FoMV-PDS-infected 
plants were examined, including nine inoculated by bombardment (plants numbered B1-B9) 
and three that were rub-inoculated (plants numbered R1-R3). Deletion of the pds insert was not 
detected in any L4 or L6 sample. However, four of 12 L9 samples contained deletions of the pds 
fragment to different extents (one minor (B5), two partial (B9, R3), and one complete (B4)), and 
11 of the L top samples had deletions of the pds fragment. The deletions were more extensive 
in the L top samples, with six of them showing PCR products consistent with complete deletion 
(B1, B2, B3, B4, R2, R3). These results corresponded well with the level of pds mRNA transcripts 
detected by qRT-PCR, with less silencing observed as the frequency and extent of deletions 
increased. When averaged over all plants, FoMV-PDS caused pds mRNA transcripts to be 
reduced to 25.4% and 27.8% of their levels in L4 and L6 empty vector control samples (Fig. 5, 
bottom-left panel). In L9, FoMV-PDS silenced pds expression by half, while pds silencing was 
further reduced in L top samples. Furthermore, when comparing rub-inoculated and 
biolistically-inoculated plants, no obvious difference was observed (Fig. 5).

Silencing of the Maize lesion mimic 22 (les22) Gene Using the FoMV Vector

To further test the ability of FoMV to silence maize genes, we targeted the lesion mimic 
gene, les22. A 329 bp fragment corresponding to the 3’ end of the les22 ORF was inserted in the 
antisense orientation at the XbaI and XhoI cloning sites in pFoMV-V. Sweet corn plants were 
inoculated with pFoMV-Les22 using biolistic bombardment. Null mutations in les22 result in the 
appearance of necrotic lesions on leaves that resemble the cell death triggered during a 
hypersensitive response to plant pathogens (Hu et al., 1998). The necrotic lesions began to 
appear at 8-10 days after inoculation with FoMV-Les22. This lesion mimic phenotype became
more obvious by two weeks after inoculation and spread to all the leaves later on (Fig. 6A).

When symptomatic leaf sections were stained with trypan blue, a histochemical assay for irreversible membrane damage indicative of dead or dying cells, blue staining was observed in brownish areas on the leaf, confirming the occurrence of cell death (Fig. 6B). These results indicate that FoMV-Les22 infection is able to induce a les22 silencing phenotype similar to that of les22 null mutants.

The lesion mimic phenotype was first observed on the 3rd leaf of FoMV-Les22-infected plants. As plants grew, the phenotype spread over all the leaves, although only tiny necrotic spots were observed on the top leaves. In most plants, the most extensive lesions were observed on leaves 4 to 6. The lesion mimic phenotype became less severe as the virus spread to upper leaves,形成a phenotypic gradient from bottom to top similar to FoMV-PDS. We tested the stability of the 329 bp Les22 insert in L4, L6, L9, and L top samples from eight FoMV-Les22 plants (Fig. 7). No deletion was detected in any of the L4 and L6 samples, partial deletions were detected in three L9 samples (B3, R1, R2), and partial or total deletions were detected in all L top samples. These results indicate that the insert was gradually lost as FoMV-Les22 moved into the upper leaves. There was no obvious difference in insert stability or les22 silencing effect between plants that were inoculated by bombardment (plants B1-B6) or rub-inoculation (plants R1-R2). Quantification of les22 mRNA levels showed that its expression was reduced to 18.1% of control in L4, 36.9% of control in L6, and 54.6% of control in L9 and no suppression was detected in L top samples (Fig.7).

Silencing iojap (ij) and brown midrib 3 (bm3) using the FoMV Vector

In addition to pds and les22, we targeted two other genes iojap (ij, Han et al., 1992) and brown midrib 3 (bm3, Vignols et al., 1995) using the FoMV vector. Loss of function mutations in ij result in variable white stripes and margin patterns on leaves (Han et al., 1992). We inserted a 231 bp fragment from near the 3’ end of the ij ORF between the XbaI and XhoI sites in pFoMV-V in the antisense orientation and the resulting construct was named pFoMV-Ij. This construct was biolistically inoculated onto sweet corn. Viral symptoms were first observed at about one week after inoculation. Several days later, white stripes were observed on leaves of infected
plants, forming white margin patterns (Fig. 8A) similar to the phenotype described for genetic mutants. QRT-PCR analysis was performed to test the expression level of \( ij \) mRNA in the 6\(^{th} \) leaf of infected plants on which obvious phenotypes were observed. These data showed that in five independent plants displaying the \( ij \) phenotype there was a significant silencing effect, because \( ij \) expression levels were only 16.7%-35.5% of non-infected controls (Fig. 8B).

In the case of \( bm3 \), the FoMV-Bm3 construct induced symptoms similar to the FoMV-V empty vector, but it did not induce an accumulation of reddish-brown pigmentation in the leaf midribs, which would be consistent with \( bm3 \) loss-of-function mutants (Barrière and Argillier, 1993). RT-PCR analysis confirmed that the 259 bp \( bm3 \) insert was present in the viral genome of systemically infected plants, and qRT-PCR analysis demonstrated that expression of the \( bm3 \) mRNA was significantly reduced to 26.5%-0.7% of the empty vector control (Supplemental Fig. S1). These data showed that \( bm3 \) was silenced even though the brown midrib phenotype was not observed.

**FoMV Infection of Maize Inbreds, Sorghum, and Green Foxtail**

We were interested to know if the FoMV infectious clone might have utility in other maize genotypes and other grass species. To test this, seedlings of sorghum, green foxtail, and 10 different inbred lines of dent corn were rub-inoculated with the FoMV-V empty vector virus. Mosaic symptoms were observed on leaves of maize inbred lines B73, B101, W22CC, K55, FR1064, B104, A188, and W64A. No viral symptoms were observed in inbred lines Mo17 and Mo47. RT-PCR further confirmed FoMV infection in systemic leaves of symptomatic lines and also in asymptomatic, systemic leaves of Mo47 plants that had been inoculated with FoMV-V. No infection was detected in 16 individual Mo17 plants inoculated with FoMV-V indicating this inbred line is resistant to our FoMV-V clone. This observation is consistent with previous work showing that Mo17 is resistant to a wild type FoMV isolate (Ji et al., 2010). FoMV-V was infectious in sorghum (Sorghum bicolor) and green foxtail (Setaria viridis) plants as evidenced by the mosaic symptoms that developed on leaves and RT-PCR results (Fig. 9). These data suggest that the FoMV vector may be useful for silencing genes in other maize genotypes and possibly in other plant species of economic and scientific interest.
To establish that FoMV VIGS is feasible in dent corn, the inbred line B73 was chosen because it is a common parent that has been utilized in generating important genetic resources such as the maize nested association mapping population (McMullen et al., 2009) and the intermated B73 x Mo17 (IBM) population (Lee et al., 2002), and it is the first and most complete maize reference genome sequence (Schnable et al. 2009). B73 seedlings were biolistically inoculated at the two-leaf stage with the pFoMV-PDS construct, and a photo-bleaching phenotype was observed on the systemic leaves at 14 dpi that was similar to sweet corn (Supp. Fig. 2A, images of L5). RT-PCR analysis demonstrated that L4 and L5 of the B73 seedlings were systemically infected by FoMV-V (empty vector) and FoMV-PDS carrying the maize pds target sequence at 14 dpi (Supp. Fig. 2B). QRT-PCR analysis showed that pds mRNA transcripts were significantly reduced in L4 and L5 of B73 plants systemically infected with FoMV-PDS at 14 dpi (Supplemental Fig. 2C). The pds mRNA transcript levels in seven silenced plants ranged from 21%-46% of the empty vector control in one replicate (Supplemental Fig. 2C), and in the second independent replicate, pds mRNA transcript levels ranged from 14%-77% of the empty vector control. In the two replicate experiments, each containing seven plants per treatment group, there was 100% efficiency for FoMV infection and pds suppression was observed in 100% of the plants infected by FoMV-PDS. These data from B73 clearly demonstrate that the FoMV VIGS system is applicable to this important dent corn inbred line.

DISCUSSION

We report the development of a DNA-based FoMV vector system for RNA silencing in maize. By using the term DNA-based, we mean that the plasmid DNA constructs are delivered directly into plant cells by biolistic inoculation, and transcription of the viral RNA is initiated in vivo by the P35S. Although a full-length infectious clone of FoMV was described by Robertson et al. (2000), there has since been no report of using FoMV as a VIGS vector to our knowledge. Disarmed FoMV vectors that cannot spread systemically have been developed previously for transient gene expression. However, those expression vectors are designed for restricted local gene expression via Agrobacterium infiltration-mediated delivery (Liu and Kearney 2010).
The FoMV vector that we have developed cannot be used to express proteins, because the foreign inserts are placed after the stop codon of ORF5. The current design of the FoMV VIGS vector allows any part of the plant gene of interest to be targeted for silencing including noncoding sequences such as promoter regions and UTRs without regard for reading frame. To illustrate this point, we elected to insert all the target gene fragments in the antisense orientation. Since we did not test gene fragments inserted in the sense orientation, there is no direct evidence that the antisense orientation provides a better silencing effect. However, our previous experience in soybean with another viral vector derived from *Bean pod mottle virus* suggests that antisense fragments may result in more extensive silencing than sense fragments (Zhang et al., 2010). Similar to FoMV, the cloning site in the *Bean pod mottle virus* was placed immediately after the stop codon following the CP coding sequence.

The insertion of the cloning site after the ORF5 stop codon was the most expeditious strategy for designing the FoMV vector. Engineering FoMV so that it could express foreign proteins as well as carry fragments of VIGS target sequences - while retaining the ability to systemically infect plants - would require creation of an additional sgRNA promoter to drive expression of the protein or VIGS target sequence. Vectors derived from PVX can be used for both VIGS and gene expression. In such PVX vectors, foreign genes or plant gene fragments for VIGS are inserted between ORF4 and ORF5 under the control of either a duplicated, native CP promoter or a heterologous CP promoter from a related *Potexvirus* (Sablowski et al., 1995; Lacomme and Chapman, 2008; Dickmeis et al., 2014; Wang et al., 2014). Utilizing a similar strategy for CP promoter duplication in FoMV is complicated by the presence of ORF5A, which overlaps with ORF4. However, a previous study suggests that ORF5A may be dispensable (Robertson et al., 2000), which could be beneficial for the promoter duplication strategy.

The PVX vectors with duplicated promoters frequently suffer partial or complete loss of inserted sequences, especially when the insert size is large or the recombinant virus is passaged (Avesani et al., 2007; Dickmeis et al., 2014). In a PVX vector study involving heterologous subgenomic promoter-like sequences, a *Bamboo mosaic virus* (BaMV) subgenomic promoter
combined with an N-terminal CP deletion resulted in the highest stability of foreign inserts following a passage to new plants (Dickmeis et al., 2014). We tested the stability of inserts in the FoMV vector at the cloning site after the ORF5 stop codon, and no deletion was detected in L4 or L6 samples, but by L9 plants began to show evidence for deletions of the inserts. This was true in both biolistically inoculated (passage 0) and rub-inoculated plants (passage 1). These observations indicate that loss of inserts become more severe at later maize developmental stages after the virus has gone through more rounds of replication. However, using L5 as a source of inoculum for rub-inoculations results in silencing phenotypes similar to biolistically-inoculated plants. The similar levels of silencing and insert stability following biotic and rub-inoculation with sap from L5 also indicate that it is feasible to passage the recombinant FoMV viruses at least one time for rub-inoculation of experimental plants, which is easier to perform and less costly in terms of time and reagents.

We have demonstrated that when the FoMV vector was used to silence pds obvious leaf photo-bleaching was observed that was consistent with pds silencing in other plant species. The mRNA transcript levels of pds were reduced by approximately 70%-80% in L4 and L6 samples in both biolistically inoculated plants and subsequent rub-inoculated plants, indicating a high efficiency of this FoMV VIGS system in certain leaves. Stable and sustainable gene silencing was also obtained using mechanical inoculation passage through leaf sap prepared from L5 of FoMV-PDS biolistically inoculated plants. In upper leaves (L9 and L top), the silencing effect was reduced, which was consistent with the deletion of the pds fragment. When les22 was targeted for silencing, necrotic spots were observed on leaves, which is a phenotype that is consistent with les22 null mutations. The spatial and temporal aspects of les22 silencing that we observed were similar to pds, indicating that the silencing effects caused by FoMV-PDS and FoMV-Les22 are typical for this version of the FoMV system. Interestingly, we did not observe the bm3 loss-of-function phenotype of the reddish-brown midribs, which was possibly due to the conditions in our growth chamber and the transient nature of our experiments. The bm3 mutant phenotype takes some time to appear in developing maize plants and it may be affected by the cells in which the down-regulation of its expression occurs (Vignols et al., 1995).
BMV was first described as a vector for gene silencing in maize in 2006 (Ding et al., 2006), and since then, only a few additional studies have reported its use for VIGS in maize (Benavente et al., 2012; Shi et al., 2011; van der Linde et al., 2011). More recently, a new CMV vector was published for VIGS in maize (Wang et al., 2016). Here, we attempt to compare and contrast FoMV to the BMV and CMV vectors with the caveat that we have no direct experience with these other two vector systems. One major difference is the preparation of the inoculum. BMV is a positive-strand tripartite RNA virus, and inoculation using the BMV vectors requires in vitro transcription of three the RNAs followed by rub-inoculation or vascular puncture inoculation (Ding et al., 2006; Benavente et al., 2012). Alternatively, the in vitro transcribed RNA mixture can be rub-inoculated onto Nicotiana benthamiana plants, and BMV virions produced in N. benthamiana are then used to rub-inoculate maize seedlings (van de Linde et al., 2011). CMV is also a positive-strand tripartite RNA virus. Its infection in maize is achieved by vascular puncture inoculation of maize kernels using crude sap from systemically infected leaves of N. benthamiana plants that had been infiltrated with an A. tumefaciens mixture containing each of the three genome segments of CMV. Rub-inoculation with the CMV-infected sap from N. benthamiana yielded a low percentage of infected maize plants (Wang et al., 2016). FoMV by contrast is a monopartite positive-strand RNA virus, so only one vector plasmid is needed, and we directly inoculate maize seedlings. It is not necessary to make in vitro transcripts, because the FoMV genome is fused to the CaMV 35S promoter, which directs the production of viral transcripts in vivo. During the period of our experiments, from 10-71% of inoculated sweet corn and 100% of B73 plants became systemically infected indicating that the inoculation procedure can be robust, but there is room to improve the consistency. We should note that the sweet corn and B73 experiments were performed in different facilities, and so environmental variables were likely to have affected the inoculation efficiencies.

A second major difference among the three maize viral vectors is the symptoms they induce. BMV causes chlorosis in infected leaves, stunted plant growth, and inhibits seed germination (Ding et al., 2006; Benavente et al., 2012). These symptoms can be moderate to severe depending on the maize line. For instance, in B73, BMV infection is so severe that it causes
plant death. Out of 30 maize lines screened, only 6 lines show moderate viral symptoms (Benavente et al., 2012). The CMV vector induces mild to moderate symptoms in maize lines characterized by leaves displaying a mosaic of striping and mottling (Wang et al., 2016). So far, we have observed FoMV infection in a sweet corn line and in 9 dent corn inbred lines and found they develop no to mild viral symptoms (Figure 9). We have not observed stunted plant growth in the lines we have tested, and we have not tested the effects of FoMV on seed germination.

\[Pds\] is a common target that was silenced using all three virus vector systems. When \(pds\) was silenced using BMV in the Va35 inbred line, maximal photo-bleaching was reported on the second systemic leaves and then decreased with each succeeding leaf (Ding et al. 2006). The number of leaves in which the photo-bleaching phenotype occurred as the plant developed and infection progressed was not reported. van der Linde et al (2011) rub-inoculated a BMV \(pds\) silencing construct onto plants at the two-leaf stage, and they reported that the strongest and most consistent photo-bleaching was observed in L6. The expression of the \(pds\) mRNA transcript was 24-81% of the empty vector control and silencing in leaves 4 and 5 was less effective and highly variable. Benavente et al (2012) inoculated the germinating maize embryo using vascular puncture inoculation (VPI), and they observed that the photo-bleaching phenotype was most common on L1-L3 and occasional on L4-L6. \(Pds\) silencing using the CMV vector was seen in the first true leaves at 18 dpi, but the effect in other leaves in later developmental stages was not shown (Wang et al., 2016). In our FoMV system, photo-bleaching of \(pds\) silencing was observed from the 3\(^{rd}\) to the 10\(^{th}\) leaf, with the majority of plants displaying photo-bleaching from the 4\(^{th}\) to the 9\(^{th}\) leaf. FoMV-PDS caused \(pds\) mRNA transcripts to be reduced to 25.4% and 27.8% of their levels in L4 and L6 compared to empty vector control samples, and in L9 \(pds\) expression was still silenced by half on average.

The FoMV viral vector system has the potential to provide a powerful biotechnological tool needed to supplement conventional genetic and transgenic plant approaches for identifying gene functions. FoMV has a broad host range including 56 species of \textit{Gramineae} (\textit{e.g.} maize, sorghum, rice, barley, green foxtail) and at least 35 dicot species (\textit{e.g.} soybean and tobacco).
(Paulsen and Niblett, 1977). We confirmed that at least nine maize inbred lines, sorghum, and green foxtail can be infected by our FoMV isolate. Thus, the successful demonstration of this FoMV vector for gene silencing in sweet corn and dent corn is expected to readily translate into a useful functional genomics platform for research and improvement in other monocot plants of economic and scientific interest.

**MATERIALS AND METHODS**

**Plants, Virus Strains, and Inoculation**

The FoMV isolate 139 used in this study was first isolated from foxtail growing in a Kansas cornfield (Paulsen and Niblett, 1977). The isolate was kindly provided by Dr. Dallas Seifers (Kansas State University). The virus was maintained in sweet corn (*Zea mays* L. ‘Golden x Bantam’; American Meadows, Williston, VT, USA). Virus-infected leaf sap was prepared by grinding infected leaves in 50 mM potassium phosphate buffer, pH 7.0. Sweet corn plants at the two-leaf stage were mechanically inoculated by rubbing leaf sap on new leaves dusted with 600-mesh Carborundum. To initiate infections from FoMV infectious clones, leaves of one week old plants were inoculated by particle bombardment using a Biolistic PDS-1000/He system (Bio-Rad Laboratories, Hercules, CA, USA), 1.0 µm gold particles coated with 1 µg of FoMV plasmid DNA, and 1,100-psi rupture disks at a distance of 6 cm. Plants were placed in the dark for 12 h before and after bombardment and then maintained in a greenhouse room with a thermostat set to 20-22 °C with a 16 h photoperiod.

**Construction of Infectious FoMV Constructs**

Unless otherwise stated, all plasmids were propagated in ElectroMax DH5α-E cells (Invitrogen, Carlsbad, CA, USA) and purified using the QiaPrep Spin MiniPrep kit (Qiagen, Valencia, CA, USA). All PCR was performed using Takara PrimeSTAR™ HS DNA Polymerase (TaKaRa Bio Inc., Otsu, Shiga, Japan). Nucleotide sequencing was done using the Big Dye Terminator DNA Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and the ABI Prism
310 genetic analyzer at the Iowa State University DNA Facility. Sequence analysis was performed using the Vector NTI program (Invitrogen, Carlsbad, CA, USA).

Total RNA extracted from FoMV-infected sweet corn leaves was used as a template for first-strand cDNA synthesis using 0.5 µg of mRNA, 0.5 µg oligo(dT)$_{20}$ primer, 1µl 10mM dNTP, and Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA) to a final volume of 20 µl. The first-strand cDNA product (2 µl) was used as template in two 100 µl PCR reactions for amplification of the 5’ and 3’ends of the FoMV genomic cDNA using the primer pairs FM-5end & FM-2388R and FM-3end & FM-2388F, respectively. The PCR conditions were as follows: 1) 1 minute at 98°C followed by three cycles of 98°C for 10 seconds, 40°C for 12 seconds, and 68°C for six and one-half minutes; 2) 30 cycles 98°C for 10 seconds, 52°C for 12 seconds, and 68°C for seven minutes; 3) 10 minutes at 68°C. The PCR products were gel extracted and used together as template in an overlap PCR reaction with primer pairs FM-5end & FM-3end for the generation of the FoMV full-length genomic cDNA. This PCR product was gel extracted, treated with T4 DNA kinase, and ligated into pSMV-NVEC (Wang et al., 2006) that had been digested with StuI and dephosphorylated to generate construct pFoMV-IA. Clones in the correct orientation were screened by PCR with primer pair FM-5501F and NosRev. Genome orientation and fidelity of pFoMV-IA was further confirmed by sequencing with primer 35-Seq and with primers spanning the FoMV genome.

To enable insertion of foreign sequences for gene silencing, a cloning site consisting of the XbaI and XhoI restriction sites was inserted just after the stop codon of the CP (Fig. 1). In PCR reaction A, primer pairs FM-5074F & FM-XbaRev were used to amplify a product from the wild type FoMV-IA infectious clone and the product was gel extracted. In PCR reaction B, primer pairs FM-XbaFor & NosRev was used with wild type infectious clone pFoMV-IA as template and the product was gel extracted. In overlap PCR reaction C, primer pair FM-5074F & NosRev was used with PCR products A & B as templates. PCR product C was digested with restriction enzymes SacII and ClaI, gel extracted, and ligated into pFoMV-IA that also had been digested with SacII and ClaI to produce the empty vector, pFoMV-V.

**Generation of FoMV Gene Silencing Constructs**
The previously generated cDNA was used for amplification of the C-terminal fragment of the maize *pds* gene (GRMZM2G410515). PCR was performed using primer pair PDSVXb and PDSVXh and the product was digested with *Xba*I and *Xho*I to insert it in the reverse orientation into pFoMV-V to generate the *pds* silencing construct, pFoMV-PDS. Similarly, fragments of the maize genes *les22* (GRMZM2G044074), *ij* (GRMZM2G004583), and *bm3* (AC196475.3_FG004) were inserted between the *Xba*I and *Xho*I sites in pFoMV-V in a reverse orientation to produce the pFoMV-Les22 and pFoMV-Ij constructs, respectively. The primers used are listed in Table 1. One-week-old maize plants were inoculated with the FoMV plasmids plus a silencing suppressor plasmid (pSMV designed to express the *Soybean mosaic virus* helper component protease) by particle bombardment. Photographs were taken two weeks after inoculation. Non-infected sweet corn plants and sweet corn infected with the empty vector were used as controls.

**RT-PCR and qRT-PCR Analysis**

Non-infected wild type leaves, pFoMV-V or pFoMV-PDS infected leaves were harvested for total RNA extraction using the RNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA). Individual leaves as indicated in figures were harvested from each plant. After first-strand cDNA synthesis, primer pair PDSrt3 and PDSrt4 was used to detect the expression of *pds* mRNA by qRT-PCR using iQ™ SYBR® Green Supermix on an iCycler real-time PCR detection system (Bio-Rad, Hercules, CA, USA). *Actin* (GRMZM2G126010) was used as an internal control with primer pair ZmActrtS and ZmActrtR. The expression level of *pds* in non-infected leaves was set as 1.0 and the delta-delta Ct method was used for comparing the expression levels of *pds* in pFoMV-V and pFoMV-PDS infected samples.

To test for the presence of insert and the silencing effect, total RNA was extracted from the leaves of FoMV-PDS, FoMV-Les22, FoMV-Ij, and FoMV-Bm3-infected sweet corn plants. RT-PCR analysis was performed to detect the presence of insert using primer pair FM-5840F and FM-6138R, and qRT-PCR was performed to test the silencing effect using primer pairs PDSrt3 and PDSrt4, Les22rtS and Les22rtA, IjrtS and IjrtA, and Bm3rtS and Bm3rtA. *Actin* was used as an internal control with primer pair ZmActS and ZmActR for RT-PCR and primer pair ZmActrtS and ZmActrtR for qRT-PCR. The expression levels of *pds*, *les22*, *ij* or *bm3* in corresponding leaves
The inoculum for rub-inoculated plants was the leaf sap prepared from the 5th leaf of the biolistically inoculated plants.

**Trypan Blue Staining**

Trypan blue staining was performed as follows. Leaf sections were submerged in trypan blue solution (25% trypan blue stock solution (0.4%), 25% lactic acid, 25% water-saturated phenol, 25% glycerol) at 70°C and infiltrated for 1 min, then placed in boiling water for 2 min, and stained overnight at room temperature. Samples were de-stained in a chloral hydrate solution (25 g in 10 ml of H₂O) followed by equilibration in 70% glycerol for observation.

**ACKNOWLEDGEMENTS**

We thank Nick Lauter for providing seed of the maize inbred lines, Mingsheng Qi for assistance with plant photography and drawing figures, and Ida Abbott for technical and greenhouse assistance. This work was supported by the Iowa State University Plant Sciences Institute and by Hatch Act and State of Iowa Funds. This is a journal paper of the Agricultural and Home Economics Experiment Station project number 3708.
Table 1. Primers used in this study

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FIGURE LEGENDS

Figure 1. Schematic representation of the Foxtail mosaic virus (FoMV) infectious clone (pFoMV-IA) with a multiple cloning site (MCS) for insertion of plant gene fragments for silencing (pFoMV-V). The MCS containing the XbaI and XhoI restriction enzyme sites was placed after the stop codon of open reading frame 5 (ORF5), which encodes the coat protein. ORF1 encodes the RNA dependent RNA polymerase and is required for replication. ORFs 2, 3, and 4 encode the triple gene block proteins required for movement. The function of ORF5A is unknown and may be dispensable. The gray bars under the viral genome indicate the viral subgenomic messenger RNAs (sgRNA1 and sgRNA2) used to express the triple gene block proteins and sgRNA 3 that expresses the coat protein. The Cauliflower mosaic virus 35S promoter (P35S) is fused to the 5’ end of the FoMV genomic RNA in order to initiate synthesis of genome-length RNA transcripts in plant cells. The viral genomic RNA terminates with a tract of A residues (Poly (A) tail), and it is followed by the nopaline synthase terminator (Tnos) in the infectious clones.

Figure 2. Infection of sweet corn (Golden x Bantam) by the Foxtail mosaic virus (FoMV) infectious clones. A. Leaf images from control and inoculated plants from left to right: non-inoculated (NI), mock-inoculated (Mock), FoMV infectious clone (pFoMV-IA), and FoMV infectious clone carrying the empty cloning site (pFoMV-V). Bar=1 cm. B. RT-PCR assay to detect the presence of FoMV in systemic leaf tissues. From left to right: non-inoculated (NI), mock-inoculated (Mock), pFoMV-IA, and leaf 6 (L6), leaf 9 (L9), and the top leaf (Ltop) of pFoMV-V inoculated plants. The 295 bp or 318 bp FoMV fragments are present in plants inoculated with pFoMV-IA or pFoMV-V, respectively. Maize actin was included as internal control for RT-PCR.

Figure 3. Virus-induced gene silencing of the maize pds gene using the FoMV vector. A. Sweet corn (Golden x Bantam) plants were biolistically inoculated with pFoMV-PDS (carries a 313 bp fragment of maize pds) infectious clone. This is an image of the fifth leaf of a FoMV-PDS-infected plant displaying the stripes of photo-bleached tissue caused by pds silencing (compare to typical mosaic symptoms of the empty vector FoMV-V-infected plants (Fig. 2A)). B. The photo-bleaching phenotype caused by pds silencing in systemic leaves of plants that were rub-
inoculated with sap from FoMV-PDS infected tissue. The photo-bleaching phenotype is shown for leaf 4 (L4) and leaf 5 (L5). Bar=1 cm.

**Figure 4.** Quantitative real-time RT-PCR analysis of *pds* expression in non-infected (NI), FoMV-V empty vector (EV), and FoMV-PDS-infected sweet corn (Golden x Bantam) plants. Significant suppression of *pds* mRNA transcripts is detected in systemic leaves of plants that were biolistically inoculated with pFoMV-PDS (* indicates P < 0.05 compared to EV, Student’s t-test). B1, B2, B3 indicates the fourth leaf of three different biolistically inoculated plants; AS1, AS2, AS3 indicates asymptomatic systemic leaves on three different biolistically inoculated plants. The *pds* silencing effect is also observed in rub-inoculated plants indicating that the FoMV-PDS can be passaged at least one time. R1-L4, R1-L5, R2-L4, R2-L5 indicates leaves four and five on two different rub-inoculated plants.

**Figure 5.** Infection course analysis of the stability of the *pds* gene fragment and *pds* mRNA silencing following biolistic or rub inoculation of FoMV-PDS in sweet corn plants (Golden x Bantam). The gel images show the RT-PCR analyses for the *pds* insert stability in FoMV-PDS infected plants. The upper gel image is the RT-PCR control showing amplification of a single maize actin mRNA fragment in all samples. The lower gel image is RT-PCR amplification across the FoMV cloning site. EV indicates the FoMV-V empty vector that carries no insert. L4, L6, L9, Ltop indicate the leaf number that the sample was taken from. The bar graphs display the relative expression level of *pds* mRNA in the indicated leaves determined by quantitative real-time RT-PCR. B# indicates the independent plants that were biolistically inoculated and the R# indicates the independent plants that were rub-inoculated. The bottom-left graph shows the relative expression of *pds* in each leaf sample type averaged over all plants. The error bars indicate standard deviation of three technical replicates for each individual plant, except for the bottom-most graph in which they indicate the standard deviation of the mean for all plants. Error bars are not indicated for the EV samples, because the expression level of each FoMV-V sample was divided by itself to obtain 1.

**Figure 6.** Silencing *les22* using the FoMV VIGS vector in sweet corn (Golden x Bantam). A. A leaf from a representative plant infected with the FoMV vector carrying a 330 bp fragment of *les22*. 
The upper panels show close-up views of sections of plants infected with mock-inoculated (mock), FoMV-V empty vector, and FoMV-Les22. The lower panels show the leaves after staining with trypan blue. Bar=0.5 cm.

**Figure 7.** Infection course analysis of the stability of the *les22* gene fragment and *les22* mRNA silencing following biolistic or rub inoculation of FoMV-Les22 in sweet corn plants (Golden x Bantam). The gel images show the RT-PCR analyses for the *les22* insert stability in FoMV-Les22-infected plants. The upper gel image is the RT-PCR control showing amplification of a single maize *actin* mRNA fragment in all samples. The lower gel image is RT-PCR amplification across the FoMV cloning site. EV indicates the FoMV-V empty vector that carries no insert. L4, L6, L9, Ltop indicate the leaf number that the sample was taken from. The bar graphs display the relative expression level of *les22* mRNA in the indicated leaves determined by quantitative real-time RT-PCR. B# indicates the independent plants that were biolistically inoculated and the R# indicates the independent plants that were rub-inoculated. The bottom-right graph shows the relative expression of *les22* in each leaf sample type averaged over all plants. The error bars indicate standard deviation of three technical replicates for each individual plant, except for the bottom-right graph in which they indicate the standard deviation of the mean for all plants. Error bars are not indicated for the EV samples, because the expression level of each FoMV-V sample was divided by itself to obtain 1.

**Figure 8.** Virus-induced gene silencing of *ij* using the FoMV vector. A. Sweet corn (Golden x Bantam) plants were mock-inoculated (left) or biolistically inoculated with pFoMV-Ij, which carries a 231 bp fragment of the maize *ij* (right). The leaf on the right (Leaf 6) shows white stripes caused by *ij* silencing. The white stripe at the leaf margin is highlighted in the red box. Bar=1 cm. B. Quantitative real-time RT-PCR analysis of *ij* expression in FoMV-V empty vector (EV) and FoMV-Ij-infected sweet corn (Golden x Bantam) plants. Significant suppression of *ij* mRNA transcripts is detected in systemic leaves of plants that were biolistically inoculated with pFoMV-Ij (* indicates P < 0.05 compared to EV, Student’s t-test). B1, B2, B3, B4 and B5 indicate the sixth leaf of five different biolistically inoculated plants.
Figure 9. FoMV infection of maize inbred lines, sorghum, and green foxtail (Setaria viridis). A. Mosaic symptoms caused by FoMV-V infection were observed on systemic leaves of maize inbred lines (B73, B101, W22CC, K55, FR1064, B104, A188 and W64A), sorghum, and green foxtail, but not on maize inbred lines Mo47 or Mo17. Bar = 1 cm. B. RT-PCR amplification of a FoMV-specific PCR product confirmed FoMV infection in maize inbred lines B73, B101, W22CC, K55, FR1064, B104, A188, W64A and Mo47, and also in sorghum and green foxtail, but not in maize inbred line Mo17 (a sweet corn sample infected with FoMV is included as a positive control). The FoMV genomic fragment can only be detected in plants inoculated with FoMV-V but not in mock-treated plants. The actin from maize, sorghum or green foxtail was included as internal control.

SUPPLEMENTAL FILES

Supplemental Figure S1. Analysis of the stability of the bm3 gene fragment in FoMV-Bm3 and bm3 mRNA silencing following biolistic inoculation of sweet corn plants (Golden x Bantam). A. RT-PCR analysis of FoMV empty vector (EV) and pFoMV-Bm3-infected sweet corn plants. Maize actin is shown in the upper panel and the FoMV-specific fragment is shown in the lower panel. Larger bands of FoMV fragments detected in pFoMV-Bm3 infected plants indicate the insertion of the 259 bp bm3 fragment in the viral genome. Numbers above the lanes indicate individual plants. B. Quantitative real-time RT-PCR analysis of bm3 expression in FoMV-V empty vector (EV), and FoMV-Bm3-infected sweet corn plants. Significant suppression of bm3 mRNA transcripts is detected in pFoMV-Bm3 infected leaves from five independent plants (Bm3-1, 2, 3, 4, 5) (* indicates P < 0.05 compared to EV, Student’s t-test).

Supplementary Figure 2. Virus-induced gene silencing of pds in maize inbred line B73 using the FoMV vector. A) B73 inbred maize plants were biolistically mock-inoculated with the helper plasmid pSMV (Mock), inoculated with pSMV and pFoMV-EV (EV), or inoculated with pSMV and pFoMV-PDS carrying a 313 bp fragment of maize pds (B1-B7). Bar = 1 cm. The 5th leaves from plants B1-B7 developed white stripes as expected for pds silencing. B) The gel images show the RT-PCR analyses for the pds insert in RNA pooled from the 4th and 5th leaves of FoMV-PDS-
infected plants. The upper gel image is the RT-PCR control showing amplification of a single maize actin mRNA fragment. The lower gel image is RT-PCR amplification across the FoMV cloning site. EV indicates the FoMV-EV empty vector that carries no insert (298 bp). Plants B1-B7 indicate FoMV-PDS-infected plants with an intact pds insert indicated by a 611 bp fragment. C) The relative expression level of pds mRNA was determined by quantitative real-time RT-PCR. Significant silencing of pds mRNA transcripts is detected in B73 plants that were biolistically inoculated with pFoMV-PDS (* indicates P < 0.05 compared to EV control; determined by ANOVA (F-test, p=0.0002; post hoc Student’s t-test). These results represent one of two independent replicates, each with seven plants.


Han CD, Coe EH Jr, Martienssen RA (1992) Molecular cloning and characterization of iojap (ij), a pattern striping gene of maize. EMBO J 11: 4037-4046


Phytopathology 67: 1346-1351


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Figure 5. Infection course analysis of the stability of the pds gene fragment and pds mRNA silencing following biolistic or rub inoculation of FoMV-PDS in sweet corn plants (Golden x Bantam). The gel images show the RT-PCR analyses for the pds insert stability in FoMV-PDS infected plants. The upper gel image is the RT-PCR control showing amplification of a single maize actin mRNA fragment in all samples. The lower gel image is RT-PCR amplification across the FoMV cloning site. EV indicates the FoMV-V empty vector that carries no insert. L4, L6, L9, Ltop indicate the leaf number that the sample was taken from. The bar graphs display the relative expression level of pds mRNA in the indicated leaves determined by quantitative real-time RT-PCR. B# indicates the independent plants that were biolistically inoculated and the R# indicates the independent plants that were rub-inoculated. The bottom-left graph shows the relative expression of pds in each leaf sample type averaged over all plants. The error bars indicate standard deviation of three technical replicates for each individual plant, except for the bottom-most graph in which they indicate the standard deviation of the mean for all plants. Error bars are not indicated for the EV samples because the expression of pds gene was not detected.
Figure 6. Silencing les22 using the FoMV VIGS vector in sweet corn (Golden x Bantam). A. A leaf from a representative plant infected with the FoMV vector carrying a 330 bp fragment of les22. Bar=1 cm. B. The upper panels show close-up views of sections of plants infected with mock-inoculated (mock), FoMV-V empty vector, and FoMV-Les22. The lower panels show the leaves after staining with trypan blue. Bar=0.5 cm.
Figure 7. Infection course analysis of the stability of the *les22* gene fragment and *les22* mRNA silencing following biolistic or rub inoculation of FoMV-*les22* in sweet corn plants (Golden x Bantam). The gel images show the RT-PCR analyses for the *les22* insert stability in FoMV-*les22*-infected plants. The upper gel image is the RT-PCR control showing amplification of a single maize actin mRNA fragment in all samples. The lower gel image is RT-PCR amplification across the FoMV cloning site. EV indicates the FoMV-V empty vector that carries no insert. L4, L6, L9, Ltop indicate the leaf number that the sample was taken from. The bar graphs display the relative expression level of *les22* mRNA in the indicated leaves determined by quantitative real-time RT-PCR. B# indicates the independent plants that were biolistically inoculated and the R# indicates the independent plants that were rub-inoculated. The bottom-right graph shows the relative expression of *les22* in each leaf sample type averaged over all plants. The error bars indicate standard deviation of three technical replicates for each individual plant, except for the bottom-right graph in which they indicate the standard deviation of the mean for all plants. Error bars are not indicated for the EV samples, because the expression level of each FoMV-V sample was divided by itself to obtain 1.
Figure 8. Virus-induced gene silencing of \( i \) using the FoMV vector. A. Sweet corn (Golden x Bantam) plants were mock-inoculated (left) or biolistically inoculated with pFoMV-i, which carries a 231 bp fragment of the maize \( i \) (right). The leaf on the right (Leaf 6) shows white stripes caused by \( i \) silencing. The white stripe at the leaf margin is highlighted in the red box. Bar=1 cm. B. Quantitative real-time RT-PCR analysis of \( i \) expression in FoMV-V empty vector (EV) and FoMV-i-infected sweet corn (Golden x Bantam) plants. Significant suppression of \( i \) mRNA transcripts is detected in systemic leaves of plants that were biolistically inoculated with pFoMV-i (\( * \) indicates \( P < 0.05 \) compared to EV, Student’s t-test). B1, B2, B3, B4 and B5 indicate the sixth leaf of five different biolistically inoculated plants.
Figure 9. FoMV infection of maize inbred lines, sorghum, and green foxtail (*Setaria viridis*). A. Mosaic symptoms caused by FoMV-V infection were observed on systemic leaves of maize inbred lines (B73, B101, W22CC, K55, FR1064, B104, A188 and W64A), sorghum, and green foxtail, but not on maize inbred lines Mo47 or Mo17. Bar = 1 cm. B. RT-PCR amplification of a FoMV-specific PCR product confirmed FoMV infection in maize inbred lines B73, B101, W22CC, K55, FR1064, B104, A188, W64A and Mo47, and also in sorghum and green foxtail, but not in maize inbred line Mo17 (a sweet corn sample infected with FoMV is included as a positive control). The FoMV genomic fragment can only be detected in plants inoculated with FoMV-V but not in mock-treated plants. The *actin* from maize, sorghum or green foxtail was included as internal control.


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