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

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Abstract
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 (Zea mays). 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 (functions in carotenoid biosynthesis), lesion mimic22 (encodes a key enzyme of the porphyrin pathway), iojap (functions in plastid development), and brown midrib3 (caffeic acid O-methyltransferase), were silenced and characterized in the sweet corn line Golden × Bantam. Furthermore, we demonstrate that the FoMV infectious clone establishes systemic infection in maize inbred lines, sorghum (Sorghum bicolor), and green foxtail (Setaria viridis), indicating the potential wide applications of this viral vector system for functional genomics studies in maize and other monocots.

Disciplines
Agricultural Science | Agronomy and Crop Sciences | Plant Breeding and Genetics | Plant Pathology

Comments
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 (*Zea mays*). 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* (functions in carotenoid biosynthesis), *lesion mimic22* (encodes a key enzyme of the porphyrin pathway), *iopj* (functions in plastid development), and *brown midrib3* (caffeic acid O-methyltransferase), were silenced and characterized in the sweet corn line Golden × Bantam. Furthermore, we demonstrate that the FoMV infectious clone establishes systemic infection in maize inbred lines, sorghum (*Sorghum bicolor*), and green foxtail (*Setaria viridis*), indicating the potential wide applications of this viral vector system for functional genomics studies in maize and other monocots.

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, 2013). 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 the ability to silence or overexpress genes in different genetic backgrounds. VIGS can knock down the 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* (Scofield et al., 2005), *Brome mosaic virus* (BMV; Ding et al., 2006), *Cymbidium mosaic virus* (CMV; Wang et al., 2016), *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*; 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 published just recently, 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 studied intensively as a model for this genus. The 6,435-kb genome of PVX contains five open reading frames (ORFs; Huisman et al., 1988). ORF1 encodes the RNA-dependent RNA polymerase, which is necessary for viral RNA replication and subgenomic mRNA (sgRNA) synthesis (Draghici et al., 2009). The overlapping ORFs ORF2, ORF3, and ORF4 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 the 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., 2000; Bruun-Rasmussen et al., 2008). Similar to PVX, the FoMV genome contains five ORFs: ORF1 expressed from the genomic RNA; ORF2, ORF3, and ORF4 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 in vivo, but it is not required for replication or for the systemic infection of plants (Robertson et al., 2000). Previously, the FoMV infectious clone was 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 infection of plants (Robertson et al., 2000). Previously, but it is not required for replication or for the systemic infection of plants (Verchot-Lubicz, 2005). The full-length genomic sequence of FoMV was obtained by two-step overlapping PCR, sites were inserted immediately after the stop codon of ORF5, which encodes the CP. ORF1 encodes the capsid protein (Fig. 1), and this clone was designated as pFoMV-IA. Inoculation of sweet corn with pFoMV-IA showed that it was infectious and induced mild mosaic symptoms that were indistinguishable from those of plants inoculated with the parental pFoMV-IA clone (Fig. 2A). The symptoms were first visible within about 1 week after biolistic inoculation and continued to develop through 2 weeks after inoculation. Under our growth conditions, sweet corn plants appear to recover from infection, because symptoms are typically observed on only the third to sixth leaves.

RESULTS

Construction of Infectious FoMV Complementary DNA Clones

The full-length genomic complementary DNA (cDNA) of FoMV was obtained by two-step overlapping PCR, and it was inserted into the pSMV-NVEC plasmid at the StuI restriction enzyme cloning site (Wang et al., 2006). This construct placed transcription of the FoMV genome under the control of the Cauliflower mosaic virus 35S promoter and the nopaline synthase terminator (Fig. 1). Clones that had the correct insert size and orientation were biolistically inoculated onto sweet corn (Golden × Bantam) to test their infectivity. One clone, designated pFoMV-IA, reproducibly infected sweet corn, 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 with 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 noninoculated and mock-inoculated plants (Fig. 2A). To further confirm FoMV infection, reverse transcription (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 noninoculated and mock-inoculated control plants (Fig. 2B).

To enable the insertion of foreign sequences into the FoMV genome, the XbaI and XhoI 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 those of plants inoculated with the parental pFoMV-IA clone (Fig. 2A). The symptoms were first visible within about 1 week after biolistic inoculation and continued to develop through 2 weeks after inoculation. Under our growth conditions, sweet corn plants appear to recover from infection, because symptoms are typically observed on only the third to sixth leaves.

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Figure 1. Schematic representation of the FoMV infectious clone (pFoMV-IA) with a multiple cloning site (MCS) for insertion of plant gene fragments for silencing (pFoMV-V). The multiple cloning site containing the XbaI and XhoI restriction enzyme sites was placed after the stop codon of ORF5, which encodes the CP. ORF1 encodes the RNA-dependent RNA polymerase and is required for replication. ORF2, ORF3, and ORF4 encode the TGB 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 mRNAs (sgRNA1 and sgRNA2) used to express the TGB proteins and sgRNA3 that expresses the CP. The Cauliflower mosaic virus 35S promoter (P35S) is fused to the 3′ end of the FoMV genomic RNA in order to initiate the 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.
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 phytocene desaturase Gene Using the FoMV Vector

To test the ability of FoMV to induce the silencing of an endogenous maize gene, we first tested phytocene desaturase (pds) because it provides a striking visual marker when its expression is reduced sufficiently to cause a photobleaching 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 XbaI and XhoI cloning sites of pFoMV-V. Sweet corn seedlings at the two-leaf stage were inoculated with the pFoMV-PDS construct by biolistic bombardment. Photobleaching was first observed at about 10 d after inoculation with pFoMV-PDS, and this phenotype became more obvious at 2 weeks after inoculation (Fig. 3A). The phenotypes caused by FoMV-PDS were observed from the third to the 10th leaf, with the majority of plants displaying photobleaching from the fourth to the ninth leaf. Among the several replications of this experiment, from 10% to 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 photobleaching phenotypes. Total RNA was extracted from the fourth leaves of these plants, and the accumulation of pds mRNA transcripts was quantified using quantitative reverse transcription (qRT)-PCR. The expression level of pds was similar between FoMV-V-infected and noninoculated 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 photobleached leaves, with transcript levels reduced from 13.5% to 27.6% of the nonsilenced 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 photobleaching phenotype. As expected, we observed that pds mRNA levels were not reduced significantly (Fig. 4, samples AS1, AS2, and 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

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Figure 2. Infection of sweet corn (Golden × Bantam) by the FoMV infectious clones. A, Leaf images from control and inoculated plants from left to right: noninoculated (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: noninoculated, mock inoculated, pFoMV-IA, and leaf 6 (L6), leaf 9 (L9), and the top leaf (Ltop) of pFoMV-inoculated plants. The 295- 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. VIGS of the maize pds gene using the FoMV vector. A, Sweet corn (Golden × Bantam) plants were biolistically inoculated with the pFoMV-PDS (carries a 313-bp fragment of maize pds) infectious clone. Shown is an image of the fifth leaf of a FoMV-PDS-infected plant displaying the stripes of photobleached tissue caused by pds silencing (compare with typical mosaic symptoms of the empty vector FoMV-V-infected plants in Fig. 2A). B, Photobleaching phenotype caused by pds silencing in systemic leaves of plants that were rub inoculated with sap from FoMV-PDS-infected tissue. The photobleaching phenotype is shown for leaf 4 (L4) and leaf 5 (L5). Bar = 1 cm.
photobleaching on two independent rub-inoculated plants. mRNA expression levels were evaluated for leaves of pFoMV-PDS-inoculated plants displaying obvious photobleaching was observed in rub-inoculated plants, indicating that FoMV-PDS can be passaged at least one time. R1-L4, R1-L5, R2-L4, and R2-L5 indicate leaves 4 and 5 on two different rub-inoculated plants. Error bars indicate the SD of three technical replicates for each individual sample.

transcripts are correlated with the photobleaching phenotype.

To test whether the silencing effect of FoMV-PDS could be passaged, the sap from leaves of the biolistically inoculated plants displaying obvious photobleaching was used to rub inoculate the first two leaves of naïve sweet corn seedlings. Photobleaching occurred after 8 to 9 d on the rub-inoculated plants, demonstrating that the ability to induce the pds silencing phenotype could be passed (Fig. 3B). Similar to the procedures described above, pds mRNA expression levels were evaluated for leaves 4 and 5 (L4 and L5) on rub-inoculated plants displaying photobleaching on two independent rub-inoculated plants. We observed that the expression of pds was reduced significantly in the photobleached 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 photobleaching on the fourth to ninth 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 photobleaching was observed on leaves 4 to 6, 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 B1–B9) and three that were rub inoculated (plants 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 and 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, and 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 graph). 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 mimic22 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 XhoI 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 to 10 d after inoculation with FoMV-Les22. This lesion-mimic phenotype became more obvious by 2 weeks after inoculation and spread to all the leaves later on (Fig. 6A). When symptomatic leaf sections were stained with Trypan Blue, a histochemical reaction can be observed (Fig. 6B).
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 third 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, forming 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, and 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 and 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; no suppression was detected in L top samples (Fig. 7).

Silencing iojap and brown midrib3 using the FoMV Vector

In addition to pds and les22, we targeted two other genes, iojap (ij; Han et al., 1992) and brown midrib3 (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 1 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 sixth 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% to 35.5% of those in noninfected 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 reduced significantly, from 26.5% to 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 (Sorghum bicolor), green foxtail (Setaria viridis), 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 that 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 and green foxtail 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 × Mo17 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 photobleaching phenotype was observed on the systemic leaves at 14 dpi in asymptomatic Mo47 plants systemically infected by FoMV-V (empty vector) and FoMV-PDS carrying the maize pds target sequence at 14 dpi (Supplemental Fig. S2B). qRT-PCR analysis showed that pds mRNA transcripts were reduced significantly in L4 and L5 of the B73 seedlings systemically infected with FoMV-PDS at 14 dpi (Supplemental Fig. S2C). The pds mRNA transcript levels in seven silenced
plants ranged from 21% to 46% of the empty vector control in one replicate (Supplemental Fig. S2C), and in the second independent replicate, pds mRNA transcript levels ranged from 14% to 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 Cauliflower mosaic virus 35S promoter. Although a full-length infectious clone of FoMV was described by Robertson et al. (2000), to our knowledge, there has since been no report of using FoMV as a VIGS vector. 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 *A. tumefaciens* 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...
untranslated regions 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 (Glycine max) 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 expedient 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 the creation of an additional subgenomic mRNA promoter to drive the 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 spp. (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 passed (Avesani et al., 2007; Dickmeis et al., 2014). In a PVX vector study involving heterologous subgenomic promoter-like sequences, a Bamboo mosaic virus 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 (passage 1) plants. These observations indicate that the loss of inserts becomes 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 those of 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 photobleaching was observed that was consistent with pds silencing in other plant species. The mRNA transcript levels of pds were reduced by approximately 70% to 80% in L4 and L6 samples in both biolistically inoculated plants and subsequent rub-inoculated plants, indicating a high efficacy of this FoMV VIGS system in certain leaves. Stable and sustainable gene silencing also was 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 le22 was targeted for silencing, necrotic spots were observed on leaves,

**Figure 8.** VIGS of ij using the FoMV vector. A, Sweet corn (Golden × Bantam) plants were mock inoculated (left) or biolistically inoculated with pFoMV-ij, which carries a 231-bp fragment of maize ij (right). The leaf at 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, Real-time qRT-PCR analysis of ij expression in FoMV-V empty vector (EV) and FoMV-ij-infected sweet corn (Golden × Bantam) plants. Significant suppression of ij mRNA transcripts is detected in systemic leaves of plants that were biolistically inoculated with pFoMV-ij (*, P < 0.05 compared with the empty vector by Student’s t test). B1 to B5 indicate the sixth leaf of five different biolistically inoculated plants. Error bars indicate the SD of three technical replicates for each individual sample.
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 those of *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 reddish-brown midribs, 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 (Shi et al., 2011; van der Linde et al., 2011; Benavente et al., 2012). More recently, a new CMV vector was published for VIGS in maize (Wang et al., 2016). Here, we attempt to compare and contrast FoMV with 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 of the RNAs followed by rub

Figure 9. FoMV infection of maize inbred lines, sorghum, and green foxtail. 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 the maize inbred lines Mo47 or Mo17. Bars = 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), sorghum, and green foxtail but not in the maize inbred line Mo17 (a sweet corn sample infected with FoMV is included as a positive control). The FoMV genomic fragment can be detected only in plants inoculated with FoMV-V but not in mock-treated plants. The *actin* gene from maize, sorghum, or green foxtail was included as an internal control.
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 der 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 inoculate maize seedlings directly. It is not necessary to make in vitro transcripts, because the FoMV genome is fused to the *Cauliflower mosaic virus* 35S promoter, which directs the production of viral transcripts in vivo. During the period of our experiments, from 10% to 71% of inoculated sweet corn and 100% of B73 plants became systemically infected, indicating that the inoculation procedure can be robust but that there is room to improve the consistency. We should note that the sweet corn and B73 experiments were performed in different facilities, 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, stunts 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 six lines showed...
The FoMV vector system has the potential to provide a powerful biotechnological tool needed to identify gene functions. FoMV has served from the third to the 10th leaf, with the majority of mental stages was not shown (Wang et al., 2016). In our 18 dpi, but the effect in other leaves in later developmental stages was not shown (Wang et al., 2016). For the FoMV isolate 139 used in this study was kindly provided by Dr. Dallas Seifers (Kansas State University). The virus was maintained in sweet corn (Zea mays var Golden × Bantam; American Maceadows). Virus-infected leaf sap was prepared by grinding infected leaves in 50 mM potassium phosphate buffer, pH 7. Sweet corn plants at the two-leaf stage were mechanically inoculated by rubbing leaf sap on new leaves dusted with 600-mesh Carbamidum. To initiate infections from FoMV infectious clones, leaves of 1-week-old plants were inoculated by particle bombardment using a Biolistic PDS-1000/He system (Bio-Rad Laboratories). 1-μm gold particles coated with 1 μg of FoMV plasmid DNA, and 1,100 p.s.i. 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°C to 22°C with a 16-h photoperiod.

Construction of Infectious FoMV Constructs

Unless stated otherwise, all plasmids were propagated in E. coli (Invitrogen) and purified using the QIAprep Spin Miniprep kit (Qiagen). All PCR was performed using Takara PrimeSTAR HS DNA Polymerase (Takara Bio). Nucleotide sequencing was done using the Big Dye Terminator DNA Sequencing Kit (Applied Biosystems) and the ABI Prism 310 genetic analyzer at the Iowa State University DNA Facility. Sequence analysis was performed using the Vector NTI program (Invitrogen).

FoMV-IA extrafloral nectary-infected sweet corn leaves was used as a template for first-strand cDNA synthesis using 0.5 μg of mRNA, 0.5 μg of oligo (dT)20 primer, 1 μL of 10 mM deoxycytidinotriphosphate, and SuperScript III reverse transcriptase (Invitrogen) to a final volume of 20 μL. The first-strand cDNA product (2 μL) was used as a template in two 100-μL PCRs for amplification of the 5′ and 3′ ends of the FoMV genomic cDNA using the primer pairs FoMV-F and FoMV-R (Wang et al., 2006) that had been digested with pSMV-NVEC (Wang et al., 2006) that had been digested with XhoI restriction sites was inserted just after the stop codon of the CP (Fig. 1). In PCR reaction A, primer pair FoMV-5F and FoMV-XbaRev was used to amplify a product from the wild-type FoMV-IA infectious clone, and the product was gel extracted. In PCR reaction B, primer pair FoMV-5F and FoMV-XbaFor and NosRev was used with wild-type infectious clone FoMV-IA as a template, and the product was gel extracted. In overlap PCR reaction C, primer pair FoMV-5F and NosRev was used with wild-type infectious clone FoMV-IA as a template, and the product was gel extracted. PCR product C was digested with restriction enzymes SacII and Clal, gel extracted, and ligated into pFoMV-IA that also had been digested with SacII and Clal 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 PDS5Va and PDS5Vb, and the product was digested with XhoI and Xhol to insert it into the reverse orientation into pFoMV-V to generate the pFoMV-PDS vector. Similarly, fragments of the maize gene les22 (GRMZM2G404047), (GRMZM2G004583), and lesion3 (AC196473_33 30 F004) were inserted between the Xhol and XhoI sites in pFoMV-V in a reverse orientation to produce the pFoMV-Les22 and pFoMV-Lj constructs, respectively. The primers used are listed in Table I. 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 protein) by particle bombardment. Photographs were taken 2 weeks after inoculation. Noninfected sweet corn plants and sweet corn plants infected with the empty vector were used as controls.

RT-PCR and qRT-PCR Analysis

Noninfected wild-type leaves and pFoMV-V- or pFoMV-PDS-infected leaves were harvested for total RNA extraction using the RNeasy Plant Mini Kit.

MATERIALS AND METHODS

Plants, Virus Strains, and Inoculation

The FoMV isolate 139 used in this study was first isolated from green foxtail (Setaria viridis) 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 var Golden × Bantam; American Maceadows). Virus-infected leaf sap was prepared by grinding infected leaves in 50 mM potassium phosphate buffer, pH 7. Sweet corn plants at the two-leaf stage were mechanically inoculated by rubbing leaf sap on new leaves dusted with 600-mesh Carbamidum. To initiate infections from FoMV infectious clones, leaves of 1-week-old plants were inoculated by particle bombardment using a Biolistic PDS-1000/He system (Bio-Rad Laboratories). 1-μm gold particles coated with 1 μg of FoMV plasmid DNA, and 1,100 p.s.i. 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°C to 22°C with a 16-h photoperiod.
Trypan Blue Staining

Trypan Blue staining was performed as follows. Leaf sections were submersed in Trypan Blue solution (25% Trypan Blue stock solution [0.4%], 25% lactic acid, 25% water-saturated phenol, and 25% glycerol) at 70°C and in lactic acid, 25% water-saturated phenol, and 25% glycerol) at 70°C and in

Supplemental Data

The following supplemental materials are available.

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).

Supplemental Figure S2. Virus-induced gene silencing of pds in maize inbred line B73 using the FoMV vector.

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LITERATURE CITED


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