Original Paper Open Access

Virus-Induced Gene Silencing in Diverse Maize Lines Using the Brome Mosaic Virus-based silencing vector

Larissa M Benavente[1], Xin Shun Ding[2], Margaret G Redinbaugh[3], Richard S Nelson[2], Peter Balint-Kurti[1,4]*

[1]US Department of Agriculture, Plant Science Research Unit, Raleigh, NC
[2]The Samuel Roberts Noble Foundation, Inc. Ardmore, OK
[3]US Department of Agriculture, Corn, Soybean and Wheat Quality Research Unit, and Department of Plant Pathology, Ohio State University, Wooster, OH
[4]Department of Plant Pathology, North Carolina State University, Raleigh NC
*Corresponding author: E-mail: Peter.Balint-Kurti@ars.usda.gov

Abstract

Virus-induced gene silencing (VIGS) is a widely used tool for gene function studies in many plant species, though its use in cereals has been limited. In addition, within cereal species the varieties that best respond during VIGS screens are often not known. Using a Brome mosaic virus (BMV) vector designed to silence the maize phytoene desaturase (PDS) gene, a genetically diverse set of maize inbred lines was screened for development of gene silencing after inoculation of seeds through the novel use of a vascular puncture inoculation technique. In addition to Va35, which previously was shown to support silencing, maize lines NC300, Ki11, Oh7b, M162W and CML52 displayed significant visible photobleaching when challenged with the BMV-PDS. In these plants, targeted PDS mRNA expression was decreased 50-80% relative to levels in plants that were inoculated with BMV containing a fragment of the GUS gene or were mock-inoculated.

Keywords: RNA silencing, VIGS, Brome Mosaic Virus, vascular puncture, gene expression, phytoene desaturase

Introduction

Maize is widely used both as a staple crop and as an advanced system for genetic research. Recent advances, including the completion of the B73 genome sequence (Schnable et al, 2009) and the development of the 5000-line nested association mapping (NAM) population (McMullen et al, 2009), provide the scientific community with much needed tools to further probe and exploit maize genetic diversity at the molecular level. One of the current deficiencies of maize as a system for genetic research is the lack of a rapid and high-throughput system to study gene function in vivo. While the production of transgenic and mutant lines is commonly used for assessing gene function in plants, producing and analyzing these lines is still a relatively laborious and time-consuming process in maize (Kodym et al, 2003; Wang et al, 2009).

Virus-induced gene silencing (VIGS) is a well-established and widely-used tool to reduce the expression of a gene of interest. VIGS exploits the plant's endogenous RNA-mediated defense system that targets viral RNAs for sequence-specific degradation (Becker and Lange, 2010; Robertson, 2004). Briefly, VIGS involves the insertion of a fragment from a plant gene of interest into the genome of a plant-infecting virus. During infection, the silencing machinery of the host recognizes and targets for destruction the host gene fragment in the virus and any host transcripts containing the same or very similar sequence. The phenotypes resulting from the reduced expression of the target transcript provide important information about the function of the targeted gene. The cellular mechanisms underlying VIGS have been reviewed previously (e.g. Llave, 2010). VIGS has been used for functional gene studies in many species (Becker and Lange, 2010; Robertson, 2004), mainly in dicotyledonous plants, although there are now workable systems for multiple members of the Poaceae, including barley (Holzberg et al, 2002), wheat (Scofield et al, 2005), oat (Pacak et al, 2010) rice (Ding et al, 2006; Purkayastha et al, 2010), maize (Ding et al, 2006), and *Brachypodium distachyon* (Demircan and Akkaya, 2010; Pacak et al, 2010). The two main systems used for VIGS in cereals, Barley stripe mosaic virus (BSMV) and Brome mosaic virus (BMV), recently have been reviewed in detail (Scofield and Nelson, 2009).

BMV is a positive-sense tripartite RNA virus and the type member of the genus Bromovirus. The BMV-based VIGS system was originally developed to fulfill the need for VIGS in a broader range of grass species (Ding et al, 2006). A BMV isolate (F-BMV) from tall fescue (*Festuca arundinacea* Schreb.) was used to construct a series of BMV-based VIGS vectors. Host range selectivity was found to be determined by F-BMV RNAs 1 and 2. In these vectors, RNA 3 was modified to carry a plant gene insert for silencing purposes. In plants infected with a combination of F-BMV RNAs 1 and 2 and an RNA 3 from a Russian strain of BMV engineered to carry a fragment of a target plant gene, accumulation of the endogenous

RECEIVED 11/15/2012

target mRNA was reduced in the infected plant tissues. In addition to barley and rice, this system was shown to effectively silence the phytoene desaturase (PDS) gene in maize cultivar Va35. More recently, this system was successfully used to assess the role of candidate genes during the interaction between the biotrophic fungus *Ustilago maydis* and Va35 maize plants (van der Linde et al, 2011) and the effect of an m-type maize thioredoxin on potyvirus infection (Shi et al, 2011). However, the BMV system confers rather transient and local gene silencing in Va35 and its efficacy across a range of maize varieties has not been reported.

The PDS gene was used as a visual reporter of VIGS in this study. PDS is an important enzyme in the carotenoid biosynthetic pathway whose silencing leads to chlorophyll photo-oxidation (Kumagai et al 1995). The ensuing photobleached phenotype is visualized as white sectors in the silenced leaf tissues (Kumagai et al, 1995), and PDS has been regularly used as a visual reporter for silencing in the development of VIGS vectors for plants (Scofield and Nelson, 2009) .

Inoculation of cereals with *in vitro*-transcribed VIGS vectors has been a concern due to cost and the potential of inefficient inoculations. The vascular puncture inoculation (VPI) procedure (Louie, 1995; Redinbaugh et al, 2001) is an efficient means for the mechanical transmission of several viruses to maize. Using a jeweler's engraving tool, the virus is delivered into the scutellum of germinating kernels by pushing minute insect pins through the inoculum and toward the vascular tissue underlying the embryo. This method can also be used to deliver cloned viral DNA (Redinbaugh et al, 2001) or *in vitro*-transcribed viral RNAs derived from cDNA clones to maize (Scheets and Redinbaugh, 2006). VPI has not been reported previously to have been used for VIGS in any plant system. Previous work on the BMV/maize system used leaf rub-inoculation (Ding et al, 2006). Potential advantages to using VPI instead of leaf rub inoculation are increased reliability of silencing and the ability to silence genes in very young seedlings. One potential disadvantage is that VPI tends to lead to systemic infection of the whole plant which may cause severe stunting and other symptoms depending on the genotype of the host.

Any tool for the investigation of gene function should ideally be applicable over a variety of maize lines. Maize is an extremely diverse species (Flint-Garcia et al, 2005). Part of this diversity has been generated by the action of Helitron transposable elements which have captured and moved genes and gene fragments from place to place within the genome such that the gene complements of two different maize lines often varies considerably (Barbaglia et al, 2011; Eichten et al, 2011; Fu and Dooner, 2002; Lai et al, 2005). Different maize lines display quite contrasting responses to BMV infection. While BMV systemically infects Va35 causing modest mosaic and streaking, in B73 it causes necrosis and plant death (Ding et al, 2001). As it stands, if an investigator's allele of interest is not present in Va35 that would preclude the use of the BMV-VIGS system to assess its putative gene function (Benavente and Scofield, 2011). Here, we build upon the BMV-VIGS system by adapting it for inoculation using VPI. This method allowed us to screen a collection of 30 diverse inbred maize lines and identify a subset of BMV-VIGS compatible maize lines.

Materials and Methods

Maize lines and plant growth conditions

Maize seeds of all the varieties used in this study were obtained from germplasm collections held by Dr. Major Goodman (Department of Crop Science, North Carolina State University). All plants were grown in growth chambers located at the North Carolina State University Phytotron. Plants were grown in chambers at 22°C with 12 h photoperiod and average light intensity of 577 µmol/m^2 s.

Virus constructs

Plasmids pF1-11 and pF2-2 were developed previously (Ding et al, 2006). Plasmid pB3m was constructed by adding 2 restriction sites (Nco I and Avr II) immediately after the coat protein open reading frame of pB3-3 via PCR. A 293 bp fragment from the maize PDS gene was amplified using the primers ZmPDS-F1 (5'-CATACCTAGGGAAATGTCTTCATAGCTGTG-3') and ZmPDS-R1 (5'-CATACCATGGCTGCAAGGCAGGATGT-3'), and cloned in the antisense orientation into the Nco I/Avr II site within the pB3m sequence to produce pB3m-PDS. Also, a 250 bp fragment was amplified from the β-glucuronidase (GUS) gene using primers GUS-F (5'-TAAGCTTCCATGGTCTTCGACCTCAATGGCGTC-3') and GUS-R (5'-TAGGCCTCCTAGGATTGCTTTGTGAGTTGCAGAGC-3'), and cloned into the pB3m to produce pB3m-GUS. The underlined sequences indicate the Nco I and Avr II restriction sites in GUS-F and GUS-R respectively. Schematic diagrams of pF1-11, pF2-2 and pB3m are shown in Supplementary Figure 1A.

To construct a modified DNA-based Brome mosaic virus gene silencing vector that synthesizes BMV RNAs 1 and 2 from the same plasmid, full length RNA1 and 2 sequences were amplified from pF1-11 and pF2-2 (Ding et al, 2006) through PCR and inserted behind a duplicated 35S promoter within a modified binary vector (Ding et al, 2010; Ding and Nelson, unpublished information). The resulting plasmid is referred to as pC13/F1+2. To construct a DNA-based vector that produces a modified BMV RNA3, two restriction sites (e.g. NcoI and AvrII) were first inserted behind the coat protein ORF in pC-BMV RNA3A/G (Ding et al, 2006) by recombinant PCR mutagenesis to create pF13m. The two added restriction sites were aids for cloning foreign inserts into the BMV sequence for gene silencing. The full length F13m se-

Table 1 - Details of primers used for qPCR.

Name	Sequence (5' to 3')	Source	qPCR efficiency
ZmPDS-F2	GGTCTATCAACAGCGAAGTATCTGGC	This work	
ZmPDS-R2	CTCCATCTTCATCCTTCCAAGCAG		1.992
ZmH2B-F1	GACATCGGCATCTCGTCC	Modified from Hachez et al, 2006	
ZmH2B-R1	GTGATGGTGGGCTTCTTG	Hachez et al, 2006	1.965
ZmEF1α-F1	ATCTGAAGCGTGGGTATGTG	Hachez et al, 2006	
ZmEF1α-R1	GCATAGCCATTGCCAATCTG	Modified from Hachez et al, 2006	2.014
ZmHATPase-F1	GGACTGCTCTGACGTACATTGAT	Hachez et al, 2006	
ZmHATPase-R1	CAGTGGCACAGGTCAAGAATCT		2.034
ZmGAPDH-F2	GACTTCCTTGGTGACAGCAGG	Modified from Hachez et al, 2006	
ZmGAPDH-R2	CTGTAGCCCCACTCGTTGTC	Hachez et al, 2006	1.982

quence was then amplified through PCR and inserted behind a double 35S promoter within the modified binary vector to yield p13A-F13m. To enhance BMV accumulation in agro-infiltrated leaves, the P19 ORF of Tomato bushy stunt virus, which encodes a silencing suppressor (Qiu et al, 2002; Voinnet et al, 1999), was amplified from pTBSV-100 (Hearne et al, 1990), the PCR product digested with XhoI and used to replace the hygromyocin gene resistance gene in p13A-F13m (Ding and Nelson, unpublished). The final DNA-based modified BMV RNA3 vector is referred to as pC13/F3-13m. Schematic diagrams of PC13/F3-13m and pC13/F1+2 are shown in Supplementary Figure 1B.

Production of infectious viral transcripts

Infectious viral RNA transcripts were made using the mMESSAGE mMACHINE T3 kit (Life Technologies, Carlsbad, CA) following the manufacturer's recommendations. Briefly, the pF1-11, pF2-2 and pB3-3-PDS and pB3m plasmids were individually digested with SpeI (pF1-11) or PshAI (pF2-2, pB3m-PDS and pB3m-GUS) for 2 h at 37°C followed by a 20 min heat-inactivation. The linearized plasmids were treated with 100 µg/ml Proteinase K at 50°C for 30 min, extracted with phenol and chloroform and resuspended in RNase-free water. For production of capped in vitro RNA transcripts, 1 µg of linearized plasmid was used as template in each 20 µl reaction, as recommended. The RNA transcripts were precipitated using lithium chloride and resuspended in RNase-free water. RNA integrity was confirmed by agarose gel electrophoresis and quantified using a Nanodrop 2000 Spectrophotometer (Thermo Scientific, Wilmington, DE). Transcripts of RNAs 1, 2 and 3 were mixed at 1:1:1 molar ratio, and then diluted in the FES inoculation buffer.

The virus containing RNAs 1, 2, and the modified RNA3 harboring the PDS insert is referred to as "BMV-PDS". Virus containing RNAs 1, 2 and the modified RNA3 carrying the GUS insert is referred to as "BMV-GUS", and is used as a non-photobleaching control virus.

Vascular Puncture Inoculation (VPI)

Pretreatment and BMV vector inoculation conditions of the kernels were as previously determined for optimal transmission of several maize viruses (Louie, 1995; Redinbaugh et al, 2001). Seeds were presoaked at 30°C in water for 2.5 h (sweet corn) or 4 h (dent corn). Dent corn was then incubated in moist paper towels at 22°C for additional 4 h. The VPI apparatus (Figure 1) consisted of five 0.2 mm stainless steel Austerlitz insect pins (Carolina Biological Supply, Burlington, NC, USA) fixed to the flattened end of 10-gauge copper wire (Louie, 1995). The assembled pins were mounted onto an engraving tool (Ideal Industries Inc, Sycamore, IL, USA). The viral RNA transcript mix (4 µl of transcript mix containing 1.5 µg of each transcript) was pipetted onto the kernel surface and this inoculum was moved 1-2 mm into the scutellum alongside the embryo toward the underlying vascular bundle using the vibrating engraving tool (Louie, 1995). Two inoculations were made approximately 1 mm from the embryo down one side of the scutellum. Subsequently, seeds were incubated for 2 days on moist paper towels at 30°C in the dark, then planted into soil and transferred to a growth chamber. Plants were inoculated with either the BMV-PDS transcript mixture, the BMV-GUS mixture or water (mock-inoculated) controls. Plants were scored for viral symptoms and leaf photobleaching up to 4 weeks post-inoculation. When screening diverse lines, four individual plants or each line were mock-inoculated, four plants were inoculated with BMV-GUS and 12 plants were inoculated with BMV-PDS.

Total RNA extraction, cDNA synthesis and Real-Time Quantitative PCR

Analysis of PDS transcript abundance was performed using real-time (RT)-quantitative PCR (henceforth referred to a qPCR). Tissue samples from photobleached sectors were harvested from leaves one through three, with surrounding green tissues being removed as much as possible. Corresponding tissues from mock-inoculated or BMV-GUS inoculated kernels were used as controls. In every case, each sample was from a different inoculated plant. Total RNA was extracted using Trizol (Life Technologies, Carlsbad, CA) as recommended. RNA integrity was

Table 2 - Lines showing photobleaching after BMV-mediated VIGS through VPI.

Line	Treatment	Germination	Virus infection	Photo bleaching
CML52	Mock	4/4	0/4	0/4
	GUS	4/4	1/4	0/4
	PDS	11/12	8/11	4/11
CML228	Mock	4/4	0/4	0/4
	GUS	5/5	5/5 ‡	0/5
	PDS	12/13	12/12 ‡	1/12
Ki11	Mock	4/4	0/4	0/4
	GUS	5/5	5/5	0/5
	PDS	12/12	7/12	4/12
Ky21	Mock	2/4	0/4	0/4
	GUS	4/4	4/4 ‡	0/4
	PDS	7/13	7/7 ‡	1/7
NC300	Mock	5/5	0/5	0/5
	GUS	9/9	6/9	0/9
	PDS	11/12	9/11	5/11
NC358	Mock	4/4	0/4	0/4
	GUS	3/4	0/3‡	0/3
	PDS	11/13	7/11‡	3/11
Oh7b	Mock	4/4	0/4	0/4
	GUS	3/4	3/3	0/3
	PDS	11/12	8/11	7/11
M162W	Mock	4/4	0/4	0/4
	GUS	4/4	0/4	0/4
	PDS	12/12	12/12	8/12
Va35	Mock	5/5	0/5	0/5
	GUS	9/10	7/8	0/8
	PDS	11/15	8/11	4/11

The ratios indicate number of seeds that germinated or displayed symptoms (mosaic for BMV-GUS and BMV-PDS treatments; or photobleaching, for BMV-PDS treatment) over the number of inoculated seeds. The virus column indicates the number of plants showing visible symptoms of viral infection over the number of total plants. The control insert (GUS) and target gene insert (PDS) were cloned into the BMV RNA 3 and inoculated with BMV RNAs 1 and 2. Mock inoculations were performed with sterile distilled water. ‡ most of the plants died over the course of the experiment.

determined by agarose gel electrophoresis and quantified using a Nanodrop 2000 Spectrophotometer. For cDNA synthesis, 1 µg total RNA was used as template in a 20 µl reaction containing 2.5 µM oligo dT(20) (Life Technologies, Carlsbad, CA), 250 ng of random primers (Life Technologies, Carlsbad, CA), 0.5 mM dNTP (Promega, Madison, WI), 10 mM DTT, and 200 U of M-MLV (Life Technologies, Carlsbad, CA), for 2 h at 37°C according to the manufacturer's recommendations. The cDNAs were then diluted to 10 ng RNA equivalent/µl using molecular biology grade water.

Sequences of all the primers used for qPCR are shown in Table 1. Quantification of the endogenous maize PDS gene was performed following standard procedures (Nolan et al, 2006). Reactions consisted of 2 µl diluted cDNA (20 ng RNA equivalent), 0.2 µM of each oligonucleotide (forward and reverse), and 1X iQ SYBR Green Supermix (Bio-Rad, Hercules, CA). Cycling conditions were: 5 min at 95°C, followed by 40 cycles of 15 s at 94°C, 30 s at 56 or 58°C and 30 s at 72°C followed by melt curve analysis as recommended by the manufacturer. A panel consisting of 10 primer pairs representing 9 reference genes (Actin, H-ATPase, EF1-α, GAPDH, Histone 2B, Polyubiquitin, α-Tubulin, γ-Tubulin,) was initially evaluated for qPCR. Some of the primers were modifications of published sequences to minimize secondary structures and primer-dimer formation. The assays were evaluated and optimized according to the following parameters: (i) presence of a single amplicon by gel and melt curve analysis; (ii) absence of primer dimers; (iii) optimization of annealing temperature, oligonucleotide concentration, and input template; (iv) qPCR efficiency between 95-105% by standard curve analysis using cDNA as template. A similar strategy was utilized for developing primers for the maize PDS sequence. Primers for the genes encoding Gapdh, Histone 2B, EF-1α, and H-ATPase were selected by these criteria and were thus used as the set of reference genes. Relative expression of the PDS transcript was calculated using the normalization factors based on the geometric mean of selected reference genes as described (Vandesompele et al, 2002). Details of all primers used are listed in Table 1. The statistical software MSTAT-C (1990, Michigan State University) was used for analysis of the RT-qPCR results. Experimental designs were completely randomized and an analysis of variance was conducted for each trial. If a significant F value was obtained indicating differences between all treatment means, then a least significant difference (lsd) value was calculated to determine which individual means differed from one other (Steel et al, 1997).

Agroinfiltration of Nicotiana benthamiana and evaluation of insert stability

The stability of inserted sequences in the BMV vector was analyzed in *N. benthamiana* using the DNA-based vector and an agroinfiltration procedure (Annamalai and Rao, 2005). *Agrobacterium tumefaciens* strain C58 was transformed with either the binary plasmid pC13/F1+2, containing BMV RNAs 1 and 2, or pC13/F3-13m, containing the engineered BMV RNA3 with a fragment of interest inserted (e.g. PDS). Transformants were maintained by culturing on LB plus rifampicin (strain selection) and kanamycin (for binary vector selection). Equal concentrations of cultures harboring the RNA1/RNA2 plasmid or the RNA3 plasmid were mixed to yield a final OD (260 nm) of 1.0. Non-transformed *A. tumefaciens* C58 was used as an inoculation control.

Three to four week old *N. benthamiana* plantlets were used for agroinfiltration. Prior to infiltration, the plantlets were transferred to a dark chamber for 16 h in a tray filled with water to induce stomata opening. The mixed cultures were then infiltrated into the abaxial leaf surface using a needless syringe until the entire leaf surface was fully infiltrated as determined

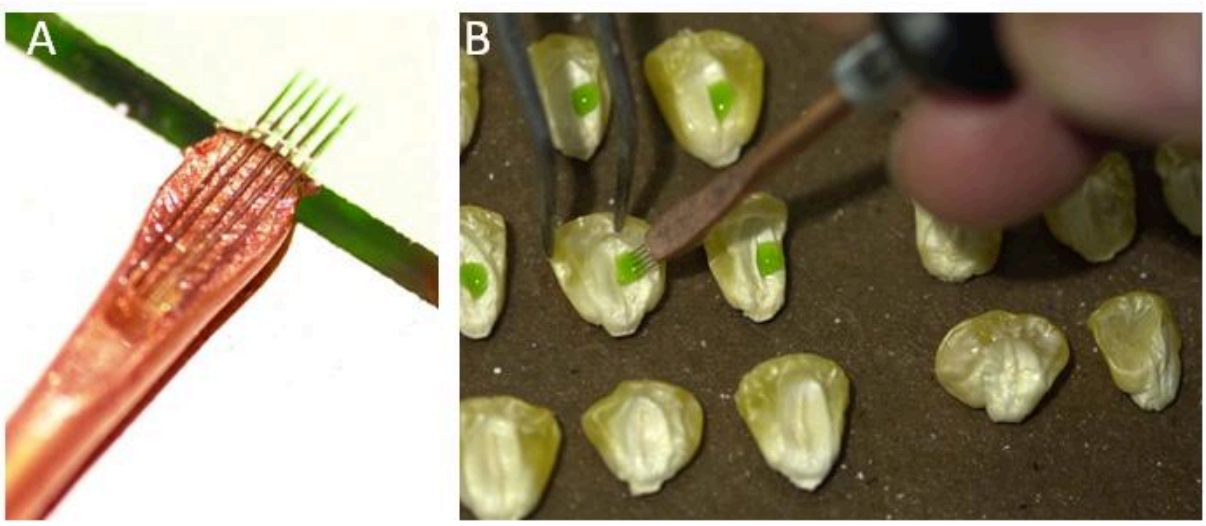

Figure 1 - (A) An example of the mounted pins used for VPI. (B) An example of the VPI procedure. Pins are held at a 45° angle form the surface of the kernel to puncture vascular tissues without injuring the embryo. This is covered in more detail elsewhere (Louie, 1995).

by visible water-soaking. Typically four plants were used per treatment. Inoculated leaves were collected from three to seven days post-infiltration (dpi) and immediately frozen in liquid nitrogen and stored at -80°C for further processing.

To detect the presence or absence of the insert in the BMV vector after inoculation, RNA was isolated from the leaf tissue using Trizol reagent as described above. RT-PCR was carried out as previously described using primers FBMV3-F1 (5'-GAATAAGGAGCTTAAGGTCGG-3') and FBMV3-R1 (5'-TGGTCTCTTTTAGAGATTTACAGTG-3' for amplification of a region containing the insert cloning site and inserted fragments in the F-BMV RNA 3 sequence. The PCR reaction buffer contained 1X buffer, 0.2 mM dNTPs, 1 mM of each oligonucleotide, 2 mM $MgCl_2$, and 0.1 µL of Taq polymerase. Amplification products were then evaluated by agarose gel electrophoresis.

Results and Discussion

The BMV-VIGS system effectively induces gene silencing in barley and rice (Ding et al, 2007). This system also has been shown to work with the maize line, Va35, but its utility in other varieties has not been reported. In order to further optimize the BMV-VIGS system for maize we had two goals.

i. Develop a robust set of inoculation procedures leading to gene silencing

ii. Screen a collection of maize inbred lines to identify those most compatible with BMV-mediated VIGS. Our initial attempts to inoculate maize with in vitro-derived transcripts of the BMV RNAs using the traditional leaf rub mechanical technique (Ding et al, 2006) yielded very low infection rates under our experimental conditions. We subsequently performed BMV-VIGS using the VPI method (Louie, 1995; Redinbaugh et al, 2001). Using this method, an infection rate above 70% was achieved and we subsequently used this technique to determine the VIGS response achieved in a panel of 30 genetically diverse maize lines including: B52, B73, CML247, Mo17, CML103, Tx303, CML333, NC358, Ki11, Oh7b, Ky21, Ki3, CML322, Oh28, CML228, B97, CML277, CML69, NC350, Oh43, CML52, Mo18W, M37W, Tzi8, M162W, Il14h, P39, H95, A632 and the sweet corn hybrid Spirit (Table 2 and data not shown). For logistical reasons, lines were tested in individual experiments involving four to six lines per experiment. NC300 was used as a positive control in each set of inoculations after initial experiments indicated strong photobleaching in leaves in this line in response to VIGS.

Inoculation of maize lines with BMV-GUS or BMV-PDS through VPI resulted in three general findings. Firstly, for lines A632, B52, B73, CM103, CML69, CML247, CML322, CML333, H95, Il14h, Ki3, NC350, Mo17, Mo18W, Oh28, Oh43, P39, Spirit, Tx303 and Tzi8, a large proportion (>50%) of inoculated seeds did not germinate. Of the seedlings that did germinate, most were severely stunted and died within 10 days, and a few grew normally (indistinguishable from the control) without BMV symptoms. Since in each case a high percentage of mock-inoculated seeds for these lines germinated, it is clear that germination was not being inhibited by the VPI procedure per se. Rather, we hypothesize that germinating seedlings of these lines were so sensitive to BMV infection that they do not emerge, and that the asymptomatic seedlings that germinated were escapes. In all cases the stunting and eventual death of the seedlings appeared to be associated with a hypersensitive type of response rather than typical viral mosaic/streaking symptoms (data not shown). This implied that it was an extreme defense response rather than disease

Figure 2 - Examples of Ki11 line plants inoculated with the BMV-PDS vector by vascular puncture inoculation. (A) 10 days and (B) 21 days after inoculation with BMV-PDS. (C) 21 days after inoculation with BMV-GUS. Comparing B and C, the difference between the bleaching caused by knockdown of PDS expression and the general streaking caused by viral symptoms can be observed.

symptoms per se that may have killed the seedlings. Secondly, for lines CML228, Ky21 and NC358, the majority of seed germinated and most plants developed severe virus symptoms, with many dying during the four week course of the experiment. Photobleaching was observed occasionally in the surviving plants (Table 2). Thirdly, for lines CML52, Ki11, M162W, NC300, Oh7b, and Va35, modest virus-induced disease symptoms (thin chlorotic strips with little lamina penetration) were observed on most plants inoculated with BMV-PDS followed by the appearance of photobleached leaves on more than half of the plants as early as 9 days post-inoculation (Table 2 and Figure 2), consistent with the VIGS visual phenotype previously described (Ding et al, 2006). For these lines, seed inoculated with BMV-GUS developed symptoms typical of the previously reported BMV infection in Va35 (Ding et al, 2006) including thin chlorotic strips and slight stunting, but no photobleaching was observed. In all experiments the mock-inoculated plants did not show virus-like symptoms or photobleaching, indicating that the photobleaching was a phenotype uniquely associated with the BMV-PDS infection.

Photobleaching phenotypes induced by BMV-PDS varied both within and between lines. This variation might be attributed to a number of factors. Seed-to-seed variation in the VPI procedure was expected due to variation in seed shape and internal morphology, and to inconsistencies in inoculation technique (e.g. duration and depth of the vascular puncture). This variation may have contributed to differences in initial infectious viral transcript load in target tissues, and may have also been the cause of the occasional examples of symptomless plants that had been inoculated with BMV-PDS or BMV-GUS. Reasons for inefficient transmission using VPI have been discussed previously (Louie, 1995; Redinbaugh et al, 2001). In addition, the physiological variations between seeds and plants at the time of inoculation and after infection will influence their response to the virus, possibly by directly influencing expression of the silencing and PDS pathway enzymes in the plant. Nonetheless, infection rates and the level of photobleaching within lines were improved and consistent enough after VPI compared with those after mechanical rub inoculations that this procedure will be useful for researchers to test phenotypes associated with silencing of specific genes in maize seedlings.

Deletion of the insert (in this case the PDS insert) directing silencing from viral genomes is a well-known feature of VIGS, especially in cereal-virus systems, and is a major cause of its transient nature (Bruun-Rasmussen et al, 2007; Ding et al, 2006; Scofield et al, 2005). If deletion of the insert occurred very early, little or no reduction in PDS expression would occur, and photobleaching would be reduced or absent. It is likely that the time after inoculation that deletion occurs varies from plant to plant in a random manner such that some plants may show extensive silencing while others, treated identically, will show extensive viral symptoms but no silencing. Time of deletion may also be influenced by the host genetic background. Consistent with this is our observation that photobleached sectors were occasionally observed in individual plants of the lines CML228, NC358, Ky21, and Oh43 on just one or two leaves on each plant (data not shown).

To further investigate the transient nature of the photobleaching phenotype and to test the hypothesis of insert instability in BMV-mediated VIGS, we utilized an agroinfiltration system to inoculate N. benthamiana, a host for BMV. In these experiments, the three BMV RNAs were inoculated to *N. benthamiana* through infiltration of Agrobacterium containing plasmids capable of transcribing full length, infectious BMV RNA genomes. The plasmid expressing BMV RNA3 contained a GUS fragment insert, allowing for analysis of its stability in the virus vector through RT-PCR analysis. For the initial experiment, we collected

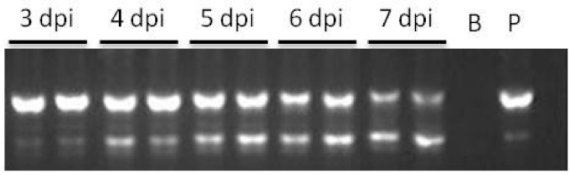

Figure 3 - RT-PCR of BMV-GUS in agroinfiltrated *N. benthamiana*. Leaves were collected at different days post-infiltration and RT-PCR was performed to determine the presence or absence of insert. Results shown below are representative of four independent biological inoculations. The upper band corresponds to an amplicon carrying insert. The smaller band corresponds to an amplicon without the insert. B indicates a no RNA control and P indicates a positive PCR control using plasmid containing the insert as a control.

leaves at 8 dpi and performed RT-qPCR using primers flanking the insertion site of the GUS fragment in the BMV RNA3 vector. We observed an almost complete loss of the insert at 8 dpi (results not shown). Subsequently we performed a more detailed analysis of insert stability, assaying for the presence of the GUS insert from 3 dpi to 7 dpi via RT-PCR (Figure 3). The larger RT-PCR product, diagnostic of an RNA3 carrying an intact GUS insert, decreased in relative abundance over time with a concurrent increase in relative abundance of a smaller product, representing an amplified fragment with no insert. The loss of insert is in concordance with results obtained with VIGS vectors in cereal species (Bruun-Rasmussen et al, 2007; Ding et al, 2006; Scofield et al, 2005). It has been suggested that both homologous and non-homologous recombination might play a role in deletions of this type (Brunn-Rasmussen et al, 2007).

Photobleaching was most commonly observed from leaves 1 to 3 (Figure 2B) and occasionally on leaves 4 to 6. The pattern of photobleaching on a leaf varied from one to many small sectors to a large area of the leaf surface. The photobleached phenotype developed mostly along the veins and spread in association with them. This was typically observed in leaves from infected Va35, NC300, and Ki11 lines. In contrast, in some plants from all BMV-VIGS compatible lines, and in many from Oh7b and M162W, the white sectors deeply penetrated the lamina, covering large sectors of the leaves. Stunting due to viral infection was observed in all varieties that showed silencing and was particularly severe in Oh7b plants (data not shown).

In order to determine if the visual photobleaching phenotype was indeed associated with the silencing of the target gene, RT-qPCR was utilized in replicated randomized experiments to quantify endogenous PDS mRNA levels in 4 maize lines (NC300, Oh7B, Ki11 and M162W) inoculated with BMV-PDS. Expression levels were normalized against four non-targeted reference genes. In the bleached sectors we observed a consistent and, for most lines, statistically significant 50-75% reduction in the endogenous PDS mRNA levels when compared with levels in corresponding mock-inoculated control plants or control plants infected with BMV carrying the GUS insert (Figure 4). For Oh7b and Ki11the the differences between mock and BMV-PDS were significant at, $p<0.05$ and for M162W and NC300, they were significant at $p<0.1$ (Figure 4). We also included in the analysis two M162W plants that displayed virus symptoms, but no photobleaching (PDSg in Figure 4). In these plants, the levels of PDS transcript were somewhat higher than the levels in the photobleached sectors, in other plants, though this difference was not statistically significant. While careful tissue harvesting was done to excise the photobleached area, most tissue samples tested included a small proportion of non-photobleached tissue. These areas may have had less silencing of the PDS transcript. Thus the level of endogenous PDS mRNA reduction in the photobleached areas may have been somewhat under-estimated. In addition, in results with other plant species undergoing VIGS, we have determined that a threshold level of PDS transcript silencing, likely at a critical developmental time point, is necessary to induce the visual photobleached phenotype (Nelson RS, unpublished).

The identification of additional maize lines that display no virus-induced systemic necrosis and visible gene silencing is a significant step forward in supplying maize researchers with germplasm for rapid functional genomic studies. Silencing phenotypes were observed in lines Va35, NC300, Oh7b, Ki11, M162W, and CML52. These lines represent a significant portion of maize diversity, including two lines (Va35 and M162W) that had been placed in separate subgroups of the “non stiff-Stalk” group, three lines (CML52, NC300 and Ki11) placed in separate subgroups of the “tropical” group and one (Oh7B) placed in the “mixed” group according to analysis based on 2039 alleles identified at 94 microsattelite loci (Liu et al, 2003). Additionally, the use of VPI was identified as a reliable and generally inexpensive method to achieve BMV infection leading to gene silencing in young tissue.

Although this report represents one step further in the optimization of the BMV-VIGS system for functional gene studies in maize, the system still has significant limitations. The main limitations are plant-to-plant variation in silencing within a line and the transient and sectored nature of the silencing phenotype due to the frequent loss of the target insert as the virus replicates. These limitations can be addressed to some extent by inclusion of appropriate controls and appropriate experimental designs (i.e., multiple replications of treatments in randomized blocks). A final significant issue to consider is that BMV inoculated through VPI induced significant stunting and viral symptoms independent of any gene silencing effects. Here we were able to readily differentiate virus symptoms from the PDS silencing phenotype (Figure 2), but these symptoms might significantly interfere with

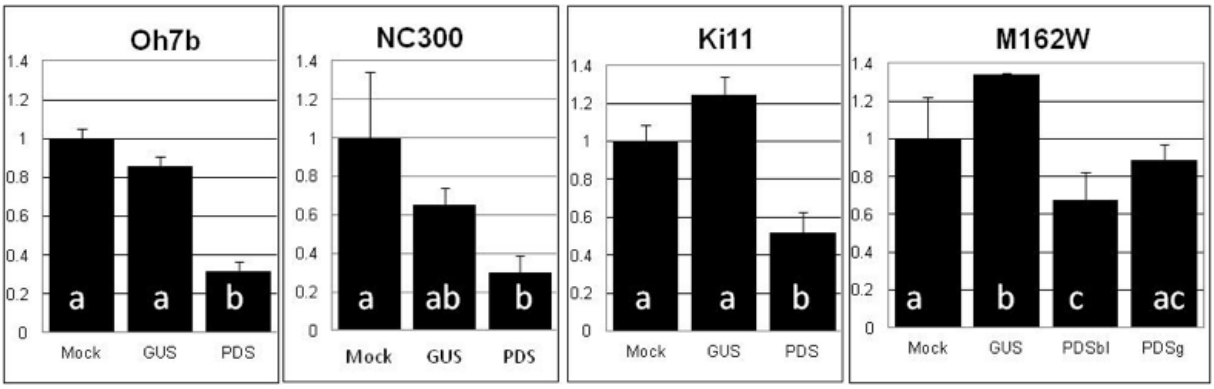

Figure 4 - Relative expression compared to mock-inoculated plants of the phytoene desaturase gene as determined by real-time RT-qPCR in three maize lines, Oh7b, Ki11,M162W. Data shown are the means ± s.d. for two to six independent plants per treatment. Mock inoculated plants were assessed as well as plants inoculated with the BMV-GUS and BMV-PDS constructs. Expression levels were normalized against a composite level for four non-targeted reference genes. Photobleached tissue was excised for assessment of PDS expression in BMV-PDS inoculated plants except for M162W PDSg in which green sectors were excised from two plants in which virus symptoms, but no photobleaching was observed. For each maize line, pairs of means, represented by columns, which do not share the same letter, indicated at the base of the column, are significantly different from one another at $p<0.1$.

the analysis of some traits. This might be particularly important when studying disease phenotypes (Tufan et al, 2011).

Acknowledgements

We thank Chris Nacci for teaching LB and PBK the VPI technique, and Kristen Willie for expert technical assistance. We thank Janet Shurtleff, Carol Saravitz and the staff of the NCSU phytotron for their expert help and use of their facilities. Dr Jose Alonso and Anna Stepanova, Department of Genetics, NCSU provided Agrobacterium strain C58. The pictures in Figure 1 were taken by Ken Chamberlain. This work was partially funded by an ARS Research Associate Program fellowship to LB and by the Samuel Roberts Noble Foundation Inc to RSN.

References

Annamalai P, Rao ALN, 2005. Replication-independent expression of genome components and capsid protein of brome mosaic virus *in planta*: A functional role for viral replicase in RNA packaging. Virology. 338: 96-111

Barbaglia AM, Klusman KM, Higgins J, Shaw JR, Hannah LC, Lal SK, 2011. Gene capture by helitron transposons reshuffles the transcriptome of maize. Genetics. Published online doi: 10.1534

Becker A, Lange M, 2010. VIGS - genomics goes functional. Trends in Plant Sci. 15: 1-4

Benavente LM, Scofield SR, 2011. A new tool for functional genomics in maize. New Phytologist. 189: 363-365

Bruun-Rasmussen M, Madsen CT, Jessing S, Albrechtsen M, 2007. Stability of barley stripe mosaic virus-induced gene silencing in barley. Mol Plant Microbe Interact 20: 1323-31

Demircan T, Akkaya M, 2010. Virus induced gene silencing in Brachypodium distachyon, a model organism for cereals. Plant Cell, Tissue and Organ Culture. 100: 91-96

Ding X, Ballard K, Nelson R, 2010. Improving virus induced gene silencing (VIGS) in rice through Agrobacterium infiltration. In: Biology of Plant-Microbe Interactions, Volume 7. Antoun, H, Avis T, Brisson L, Prevost D, Trepanier M, eds. International Society for Molecular Plant-Microbe Interactions, St. Paul, MN

Ding XS, Boydston CM, Nelson RS, 2001. Presence of brome mosaic virus in barley guttation fluid and its association with localized cell death response. Phytopathology 91: 440-448

Ding XS, Rao CS, Nelson RS, 2007. Analysis of gene function in rice through virus-induced gene silencing. Methods Mol Biol 354: 145-60

Ding XS, Schneider WL, Chaluvadi SR, Mian MA, Nelson RS, 2006. Characterization of a brome mosaic virus strain and its use as a vector for gene silencing in monocotyledonous hosts. Mol Plant Microbe Interact 19: 1229-39

Eichten SR, Foerster JM, de Leon N, Kai Y, Yeh C-T, Liu S, Jeddeloh JA, Schnable PS, Kaeppler SM, Springer NM, 2011. B73-Mo17 near-isogenic lines demonstrate dispersed structural variation in maize. Plant Physiology 156: 1679-1690

Flint-Garcia SA, Thuillet AC, Yu J, Pressoir G, Romero SM, Mitchell SE, Doebley J, Kresovich S, Goodman MM, Buckler ES, 2005. Maize association population: A high-resolution platform for quantitative trait locus dissection. Plant J 44: 1054-64

Fu H, Dooner H-K, 2002. Intraspecific violation of genetic colinearity and its implications in maize. Proc Nat Acad Sci USA 99: 9573-9578

Hachez C, Moshelion M, Zelazny E, Cavez D, Chau-

mont Fo, 2006. Localization and quantification of plasma membrane aquaporin expression in maize primary root: A clue to understanding their role as cellular plumbers. Plant Molecular Biology. 62: 305-323

Hearne PQ, Knorr DA, Hillman BI, Morris TJ, 1990. The complete genome structure and synthesis of infectious RNA from clones of tomato bushy stunt virus. Virology 177: 141-151

Holzberg S, Brosio P, Gross C, Pogue GP, 2002. Barley stripe mosaic virus-induced gene silencing in a monocot plant. Plant J 30: 315-27

Kodym A, Afza R, Grotewold E, 2003. Physical and chemical mutagenesis, pp. 189-203. In: Plant Functional Genomics, vol 236. Walker JM ed. Humana Press

Kumagai MH, Donson J, della-Cioppa G, Harvey D, Hanley K, Grill LK, 1995. Cytoplasmic inhibition of carotenoid biosynthesis with virus-derived RNA. Proc Nat Acad Sci USA 92: 1679-1683

Lai J, Li Y, Messing J, Dooner HK, 2005. Gene movement by helitron transposons contributes to the haplotype variability of maize. Proc Nat Acad Sci USA 102: 9068-9073

Liu K, Goodman M, Muse S, Smith JS, Buckler E, Doebley J, 2003. Genetic structure and diversity among maize inbred lines as inferred from DNA microsatellites. Genetics 165: 2117-2128

Llave C, 2010. Virus-derived small interfering RNAs at the core of plant-virus interactions. Trends Plant Sci 15: 701-707

Louie R, 1995. Vascular puncture of maize kernels for the mechanical transmission of maize white line mosaic virus and other viruses of maize. Phytopathology 85: 139-143

McMullen MD, Kresovich S, Villeda HS, Bradbury P, Li HH, Sun Q, Flint-Garcia S, Thornsberry J, Acharya C, Bottoms C, Brown P, Browne C, Eller M, Guill K, Harjes C, Kroon D, Lepak N, Mitchell SE, Peterson B, Pressoir G, Romero S, Rosas MO, Salvo S, Yates H, Hanson M, Jones E, Smith S, Glaubitz JC, Goodman M, Ware D, Holland JB, Buckler ES, 2009. Genetic properties of the maize nested association mapping population. Science 325: 737-740

Nolan T, Hands RE, Bustin SA, 2006. Quantification of mRNA using real-time RT-PCR. Nat Protoc 1: 1559-82

Pacak A, Geisler K, Jorgensen B, Barciszewska-Pacak M, Nilsson L, Nielsen T, Johansen E, Gronlund M, Jakobsen I, Albrechtsen M, 2010. Investigations of barley stripe mosaic virus as a gene silencing vector in barley roots and in brachypodium distachyon and oat. Plant Methods 6: 26

Purkayastha A, Mathur S, Verma V, Sharma S, Dasgupta I, 2010. Virus-induced gene silencing in rice using a vector derived from a DNA virus. Planta 232: 1531-40

Qiu W, Park J-W, Scholthof HB, 2002. Tombusvirus p19-mediated suppression of virus-induced gene silencing is controlled by genetic and dosage features that influence pathogenicity. Mol Plant-Microbe Interac15: 269-280

Redinbaugh MG, Louie R, Ngwira P, Edema R, Gordon DT, Bisaro DM, 2001. Transmission of viral RNA and DNA to maize kernels by vascular puncture inoculation. Journal of Virological Methods 98: 135-143

Robertson D, 2004. VIGS vectors for gene silencing: Many targets, many tools. Annual Rev Plant Biol 55: 495-519

Scheets K, Redinbaugh MG, 2006. Infectious cdna transcripts of maize necrotic streak virus: Infectivity and translational characteristics. Virology 350: 171-183

Schnable PS, Ware D, Fulton RS, Stein JC, Wei F, Pasternak S, Liang C, Zhang J, Fulton L, Graves TA, 2009. The b73 maize genome: Complexity, diversity, and dynamics. Science 326: 1112-1115

Scofield SR, Huang L, Brandt AS, Gill BS, 2005. Development of a virus-induced gene-silencing system for hexaploid wheat and its use in functional analysis of the Lr21-mediated leaf rust resistance pathway. Plant Physiol 138: 2165-73

Scofield SR, Nelson RS, 2009. Resources for virus-induced gene silencing in the grasses. Plant Physiol 149: 152-157

Shi Y, Qin Y, Cao Y, Sun H, Zhou T, Hong Y, Fan Z, 2011. Influence of an m-type thioredoxin in maize on potyviral infection. Eur J Plant Pathol 131: 317-326

Steel RGD, Torrie JH, Dickey DA, 1997. In: I. The Principles and procedures of statistics. A biometrical approach. 3rd edition, McGraw-Hill companies

Tufan HA, Stefanato FL, McGrann GRD, MacCormack R, Boyd LA, 2011. The barley stripe mosaic virus system used for virus-induced gene silencing in cereals differentially affects susceptibility to fungal pathogens in wheat. J Plant Physiol 168: 990-994

van der Linde K, Kastner C, Kumlehn J, Kahmann R, Doehlemann G, 2011. Systemic virus-induced gene silencing allows functional characterization of maize genes during biotrophic interaction with ustilago maydis. New Phytologist 189: 471-483

Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, Speleman F, 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol 3: 0034 37

Voinnet O, Pinto YM, Baulcombe DC, 1999. Suppression of gene silencing: A general strategy used by diverse DNA and RNA viruses of plants. Proc Nat Acad Sci USA 96: 14147-14152

Wang K, Frame B, Ishida Y, Komari T, Bennetzen JL, Hake S, 2009. Maize transformation, pp. 609-639. Handbook of Maize. Bennetzen JL, Hake SC eds. Springer, New York