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Barley stripe mosaic virus (BSMV) as a virus-induced gene silencing vector in maize seedlings

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Abstract
Barley stripe mosaic virus (BSMV) was the first reported and still widely used virus-induced gene silencing (VIGS) vector for monocotyledons including wheat and barley. Despite BSMV's reported infectivity on maize (*Zea mays*), the use of the virus as a vector in maize has not been optimized. Here, we assayed infectivity of BSMV in different maize cultivars by vascular puncture inoculation. Through knockdown of the endogenous host phytoene desaturase gene, we demonstrate for the first time that BSMV can be used as a VIGS vector in maize. This adds BSMV to the repertoire of tools available for functional studies in maize.

Keywords Barley stripe mosaic virus (BSMV) · Virus-induced gene silencing (VIGS) · Maize · Vascular puncture inoculation (VPI) · Phytoene desaturase (PDS)

The 2.4-gigabase genome sequence of the ten maize chromosomes was first published for B73 maize [1], and additional, diverse maize sequences have since been reported [2–4]. The functional roles of the majority of the predicted 39,498 genes are yet to be characterized. Among the different functional genomic tools currently available, virus-induced gene silencing (VIGS) has great promise, especially in monocot genomes with extensive gene sequence duplication where functional assessment by mutagenesis is difficult. Despite the agricultural value of monocotyledonous crops, fewer effective VIGS vectors are available than for dicotyledonous plants [5–7]. Among the different viral vectors available for maize, so far Brome mosaic virus (BMV) [8–13], Foxtail mosaic virus (FoMV) [14], and Cucumber mosaic virus (CMV) [15] have been successfully adapted as VIGS vectors. Barley stripe mosaic virus (BSMV; family *Virgaviridae*, genus *Hordeivirus*) has been one of the most widely used gene silencing vectors for monocotyledonous crops including barley and wheat [16–23]. Even though some maize genotypes, such as Oh28, are hosts of BSMV [24], BSMV VIGS has not been reported for maize to our knowledge. We show that the agro-binary vector-based infectious cDNA clone of BSMV isolate ND18 [23], kindly provided by Dr. Dawei Li, China, can also be used for VIGS in maize plants that are inoculated by vascular puncture inoculation (VPI) [25, 26].

To test the infectivity of BSMV in maize, binary plasmids pCaBS-α, pCaBS-β, and pCaBS-γ [23] expressing each of the three genomic segments of BSMV (α, β, and γ) were transformed into C58C1 *Agrobacterium tumefaciens*. Transformed cultures containing these and an expression construct (pCASS4N-p19) for the known suppressor of RNA silencing from Tomato bushy stunt virus (TBSV) p19 were prepared for inoculation as reported previously [27]. The binary plasmid construct pCASS4N-p19, kindly provided by Dr. Siddarame Gowda (University of Florida), carries TBSV-p19 gene under 35S promoter and TEV leader sequence [28, 29]. Agrocultures adjusted to 0.8 OD (600 nm) were mixed in a 1:1:1:2 ratio (α:β:γ:p19) and infiltrated into the

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abaxial surface of 3- to 4-week-old *Nicotiana benthamiana* plants. Five days post inoculation (dpi), infiltrated leaf tissue of *N. benthamiana* was ground in 0.01M potassium phosphate buffer (pH 7.0) (5 ml buffer per gram of tissue), centrifuged for 1 min at 10,000 rcf (relative centrifugal force), and used to inoculate maize. VPI inoculation methodology was used to inoculate sterile water-soaked maize kernels as described previously [25, 26]. A panel of six maize genotypes, comprising genotypes with broad susceptibility to maize viruses (Spirit, B73, Oh28, Wf9xOh51a) and strong potyvirus and multi-virus resistance (Oh1VI, Pa405) [30, 31], were used for inoculations. Three near-isogenic lines with Oh28 genetic background carrying introgressed *Wsm* alleles conferring resistance against potyviruses from the cultivar Pa405 [32, 33] were also tested by VPI with BSMV.

The potential utility of BSMV for VIGS in maize was evaluated by targeting the maize phytoene desaturase gene. An 81-nt maize PDS sequence (corresponding to nt 815–895 of GenBank accession no. L39266.1) was inserted in the γ segment of BSMV (γPDS). To do this, sequence SP230 (5′-CGTAGCTTCTTCTTTTGAAGATTCTGTTGGATGTGATGATTCTTCTTCCGTTTCTAAGAGGCCT*TTCCTGATCGGGTGAACGATGAGGTTTTTATTGCAATGTCCAAGGCACTCAATTTCATAAATCCTGATGAGCTATCTATGC*GGCGCGCCGGGCCCGGTGGTGGTGGTTAAAAAAAAAAAAAAATGTTTGATCAGATCATTCAAATCTGATGGTG-3′) was synthesized in vitro (IDT DNA technologies) to contain sequence corresponding to BSMV-γ nts 2479–2536 and 2537–2583 (U13917.1), an 81-bp maize PDS insert sequence (italicized; nt 815–895 of L39266), and pCa-γbLIC (modified pCaBS-γ with restriction sites) [23] with restriction sites (*Stu*I, *Asc*I, and *Apa*I; underlined). A virus-specific fragment corresponding to nt 2029–2529 of BSMV γ (GenBank accession no. U13917.1) was amplified with the primers SP223 (5′-CGAGTGGTGAACTCTAGGTCC-3′) and SP224 (5′-ACGGAAGAAGAATCATCACATC-3′). The two overlapping fragments were first joined by PCR with the primers SP223 and SP226 (5′-CAAACATTTTTTTTTTTTTTAACCACCACCACCGGGCCC-3′). The gel-purified PCR product of the two joined fragments was then inserted by Gibson assembly [34] into the pCa-γbLIC cut with *Kpn*I and *Apa*I.

BSMV (α + β + γ + p19) and BSMV-PDS (α + β + γPDS + p19) infection rates, scored by symptom development, differed among cultivars (Table 1). BSMV symptoms and average infection rates of 30–57% were observed in inoculated Spirit, B73, Oh28, and Wf9 x Oh51a genotypes, with similar infection rates for BSMV-PDS constructs. However, in contrast to previous reports of BSMV-susceptible maize genotypes including Oh28

Table 1 Infectivity of Barley stripe mosaic virus (BSMV) wild-type and PDS-containing cDNA constructs by vascular puncture inoculation (VPI; [25]) of maize kernels and photobleaching (*VIGS* virus-induced gene silencing) by the BSMV-PDS construct

Cultivar	Exp. I			Exp. II			Exp. III			Exp. IV			Exp. V			Exp. VI			Exp. VII			**BSMV-WT Total Sym.**	**BSMV-PDS VIGS Total Sym.**	**Total VIGS**
	WT Sym.[a]	Sym.	VIGS	WT Sym.	Sym.	VIGS	WT Sym.	Sym.	VIGS	WT Sym.	Sym.	VIGS	WT Sym.	Sym.	VIGS	WT Sym..	Sym.	VIGS	WT Sym.	Sym/	VIGS			
Spirit	13/22	13/24	5/24	6/21	8/26	2/26	22/68	31/103	16/103	5/8	3/9	3/9	2/25	1/3	0/3	2/25	2/22	0/22	5/10	8/15	2/15	**66/202 (33%)**	**66/202 (33%)**	**56/202 (28%)**
Pa405	1/5	0/11	0/11	0/24	0/29	0/29	0/15	--	--	2/3	0/5	0/5	4/22	0/5	0/5	4/22	2/13	0/13	1/15	0/14	0/14	**8/94 (8.5%)**	**2/77 (2.6%)**	**0/77 (0.0%)**
OhVI	0/9	0/22	0/22	0/23	0/27	0/27	5/24	--	--	3/24	1/20	0/20	0/27	0/14	0/14	0/27	0/30	0/30	0/22	0/25	0/25	**9/152 (5.9%)**	**5/138 (3.6%)**	**0/138 (0/0%)**
Oh28	4/18	4/17	2/17	14/26	8/29	4/29	18/25	--	--	18/19	13/20	5/20	19/26	14/19	6/19	19/26	20/26	10/26	4/17	10/23	1/23	**80/153 (52%)**	**69/134 (52%)**	**28/134 (21%)**
B73	4/10	16/34	6/34	23/30	22/29	6/29	14/26	--	--	NG[b]	NG	NG	3/41	1/3	0/3	3/41	0/36	0/36	1/24	0/23	0/23	**31/134 (23%)**	**39/125 (31%)**	**12/125 (9.6%)**
Wf9xOh51a	5/8	11/29	2/29	11/27	0/29	0/29	13/14	--	--	13/13	12/15	3/15	19/29	4/26	0/26	19/29	14/27	5/27	10/25	9/20	2/20	**85/140 (61%)**	**44/146 (30%)**	**12/146 (8.2%)**
Wsm1 NIL	3/13	5/19	1/19	--	--	--	--	--	--	NG	2/2	0/2	0/8	NG	NG	0/8	6/12	1/12	0/4	NG	NG	**3/25 (12%)**	**13/33 (39%)**	**2/33 (6.1%)**
Wsm2 NIL	8/19	16/21	5/21	--	--	--	--	--	--	0/3	0/5	0/5	10/21	2/7	0/7	10/21	1/11	0/11	2/14	8/16	1/16	**20/79 (25%)**	**27/60 (45%)**	**6/60 (10%)**
Wsm3 NIL	0/11	2/12	0/12	--	--	--	--	--	--	4/14	1/9	0/9	1/16	2/16	1/16	1/16	0/19	0/19	1/15	0/7	0/7	**8/71 (11%)**	**5/63 (7.9%)**	**1/63 (1.6%)**

Sym. = Symptomatic

[a]Gray cells show values for wild-type BSMV construct, while other cells show values for BSMV-PDS construct

[b]NG = No growth

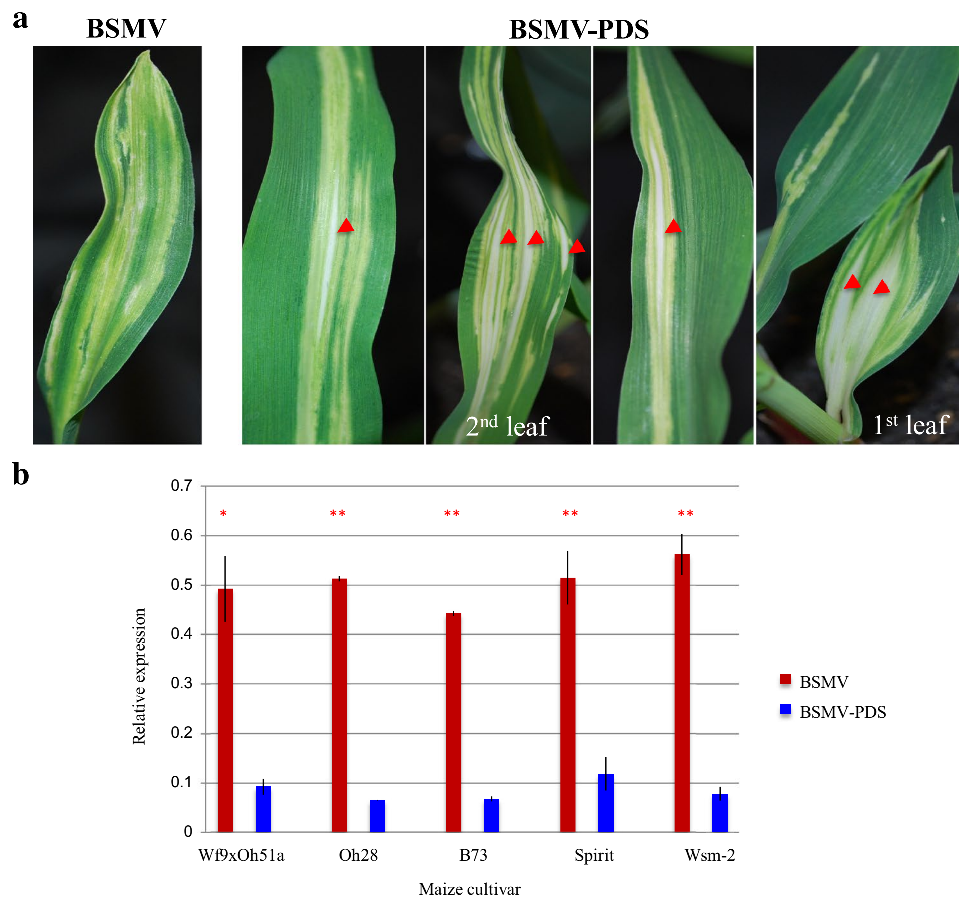

Fig. 1 Barley stripe mosaic virus-induced gene silencing in maize. **a** Images of maize (cv. Spirit) seedlings VPI inoculated with 5 dpi leaf extracts of *N. benthamiana*, agro-infiltrated with BSMV (α, β, and γ) and BSMV-PDS (α, β, and γ-zmPDS) clones. All leaves are shown at 12 days post VPI (dpv). The PDS gene silenced areas of the leaves appeared as long parallel white-colored or photobleached streaks distinct from the chlorotic greenish yellow symptoms observed with wild-type virus inoculations indicated with red arrowheads. **b** PDS mRNA levels measured by SYBR Green RT-qPCR in different maize cultivars inoculated with BSMV or BSMV-PDS. Symptomatic leaf tissue from BSMV-inoculated plants and photobleached leaf tissue from BSMV-PDS-inoculated plants were collected at 11–13 dpv. Approximately 400 ng of total RNA was used for cDNA preparation with oligo dT primers using SuperScript III First-Stand cDNA Synthesis kit (Invitrogen, Carlsbad, CA), as per the manufacturer's protocol. Primers zmPDS-F 5′-GTACGAGACTGGGCTTCATAT-3′ and zmPDS-R 5′-TGGCATGGCGAATATCATAGAG-3′ were used to measure PDS mRNA levels with SYBR Green RT-qPCR (Bio-Rad; SsoAdvanced Universal SYBR Green Supermix). RT-qPCR analysis was conducted on three plants of each treatment, with each plant sample replicated in triplicate in analyses as a control for pipetting error. Total RNA from each sample was extracted and 20 ng used for cDNA synthesis. The maize PDS gene expression was measured by amplification with specific primers (zmPDS-F and zmPDS-R; primer efficiency $e = 100\%$) compared to the reference gene folylpolyglutamate synthase (FPGS; primers FPGS-F and FPGS-R; $e = 102\%$) [38]. Primer amplification efficiency values were estimated based on the standard curves developed through amplification of serially diluted plasmid DNA clones of respective amplicons. Corrected Ct values were generated by multiplying the arithmetic mean Ct value of biological and technical replicates of each treatment to the log base two of amplification efficiency value [39, 40]. The data represent results from two biological replicates with three technical replicates for each biological replicate. Samples were collected from tissue showing photobleaching for BSMV-PDS samples. The significance in difference based on t test; p values 0.05 and 0.001 are shown, respectively, with single (*) and double (**) asterisks. Vertical error bars represent standard error deviation. (Color figure online)

[24, 35–37], the symptoms observed did not extend beyond the fifth leaf in the vascular puncture-inoculated plants we tested. The multi-virus-resistant cultivars Pa405 and Oh1VI [30, 31] exhibited lower susceptibility to BSMV, with the average infection rates of 1–12%. *Wsm* NIL had lower survival/overall germination, confounding conclusions about BSMV susceptibility, but with trends indicating some possible resistance conferred by *Wsm3*. In contrast, these alleles show resistance strength in a *Wsm1* > *Wsm2* > *Wsm3* pattern for potyviruses [32, 33]. Mechanical rub-inoculations of 7-day-old seedlings of the same maize genotypes with extracts from agro-inoculated *N. benthamiana* resulted in few infections (data not shown), despite systemic infections reported in Oh28 and other maize genotypes by others [24, 35–37], suggesting that other conditions or virus genotypes may improve BSMV infection in maize.

BSMV-PDS (α + β + γPDS + p19) clones inoculated to maize by VPI produced the expected VIGS photobleaching phenotype in the first and second leaves in a subset of symptomatic plants (Fig. 1a). The photobleaching symptoms appeared along midribs as long parallel white-colored streaks distinct from the light greenish yellow and occasionally necrotic symptoms observed with wild-type BSMV. The average percentage of plants showing photobleaching varied from 7 to 21% among the susceptible genotypes, whereas the uninfected genotypes showed no photobleaching phenotype (Table 1). PDS mRNA levels were measured by quantitative reverse transcription polymerase chain reaction (RT-qPCR). Silenced tissue showed three- to eightfold decrease in PDS mRNA levels compared to chlorotic tissues from wild-type virus inoculations (Fig. 1b).

The BSMV was developed as a VIGS vector in 2002 [19]. Since then, the virus has been used in a number of studies for functional characterization of different genes in monocots. Despite the widespread use of the virus and its reported infectivity in maize, the virus has not previously been reported to have utility as a VIGS vector in maize. Here we show that BSMV can be readily inoculated into maize through VPI and the virus can be used to induce gene silencing in maize seedlings. Like other current VIGS vectors, this approach to utilize BSMV in maize seedlings has disadvantages of virus symptoms, low infection rates, and patchy phenotypic penetrance. These limitations are not unique to BSMV, but similar observations were made with other VIGS vectors such as BMV [8, 41]. Being one of the commonly used vector systems, BSMV offers advantages in terms of better understanding of its molecular biology and opportunities for further improvements with additional research efforts. Agro-infiltration in *N. benthamiana* followed by VPI inoculations of maize kernels has been the method of choice for using gene silencing vectors in maize [4, 8]. The VPI methodology allows inoculation of many plants with minimal inoculum. Some of the challenges such as low infection rates can be circumvented through VPI inoculations with large sample size and potentially through simultaneous silencing of a gene of interest and a phenotypic marker gene such as PDS. By reducing the virus-specific symptoms through sequence modifications in viral cDNA clone and with further optimization of conditions to improve silencing spread, BSMV may be adapted as a useful VIGS vector for maize seedlings, adding the virus to the repertoire of tools available for functional studies in maize. Comparative characterization of VIGS vectors has not been done so far. Now with the advent multiple VIGS vectors for maize, including BMV, BSMV, CMV, and FoMV, relative characterization studies would provide better understanding of the challenges and opportunities conferred by these different vectors in maize functional genomic studies.

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Author contributions Project research was carried out by SJ under the direction of LS, with technical support and analyses by KW. All authors contributed to and approved the manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

Research involving human and animal participants This article does not contain any studies with human participants or animals performed by any of the authors.

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