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BRIEF REPORT

Soil-borne wheat mosaic virus infectious clone and manipulation for gene-carrying capacity

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Abstract A full-length infectious cDNA clone of soil-borne wheat mosaic virus (SBWMV; genus *Furovirus;* family *Virgaviridae*) was developed for agrobacterium delivery. The cloned virus can be agroinfiltrated to *Nicotiana benthamiana* for subsequent infection of wheat (*Triticum aestivum*, L.). The utility of the virus as a vector for gene silencing and expression was assessed through sequence insertions in multiple sites of RNA2. Virus-induced photobleaching was observed in *N. benthamiana* but not in wheat, despite the stability of the inserts. The SBWMV infectious clone can be used for further studies to investigate the biology of SBWMV through mutagenesis.

Keywords SBWMV · *Furovirus* · Infectious clone · Gene silencing

Soil-borne wheat mosaic virus (SBWMV), the type member of the genus *Furovirus* (family *Virgaviridae*), causes yellow mosaic disease in winter wheat (*Triticum aestivum* L.) [12]. SBWMV is transmitted by *Polymyxa graminis* Ledingraham [8]. The two single-stranded positive-sense RNA genomic segments are separately encapsidated in rigid rod-shaped virions. The 7.1-kb RNA1 encodes three proteins: the 150-kilodalton (kDa) and 209-kDa read-through replicase proteins and the 37-kDa movement protein (MP). The 3.6-kb RNA2 encodes three proteins: the 19-kDa coat protein (CP), the larger 84-kDa CP read-through protein (CPRT), and the 19-kDa cysteine-rich protein (CyR) (Fig. 1A) [19]. Studies on furovirus molecular biology to date have focused primarily on Japanese soil-borne wheat mosaic virus (JSBWMV; formerly classified as SBWMV-JT) because of the availability of infectious clones [21]. Development of full-length cDNA clones of SBWMV were less successful due to the instability of RNA1 in *Escherichia coli*, but one was developed using transcripts prepared from two overlapping RNA1 cDNA products [13]. We describe the development of an agro-binary plasmid-based full-length infectious clone of an Ohio SBWMV isolate and evaluation of multiple insertion sites within RNA2 for gene-carrying capacity.

Ohio soft red winter wheat samples collected in spring 2012 were used for RNA-Seq to identify virus sequences as described previously [20]. SBWMV 7105-nt RNA1 and 3593-nt RNA2 genomic sequences were amplified and confirmed by Sanger sequencing (MCIC, Wooster, OH; GenBank accession numbers KT736088 and KT736089). This isolate showed 99-100 % nucleotide sequence identity to partially sequenced isolates from New York (AY016007.1, AY016008.1, AF361641.1 and AF361642.1), Illinois (HM133584.1, HM133583.1 and AB002812.1) and Germany (JX468081.1 and JX468082.1) but only 89-90 % identity to the type strain of SBWMV (Nebraska isolate; L07937.1 and L07938.1). Despite their low nucleotide sequence similarity, the Ohio and type SBWMV isolates have 100 % identical predicted CP amino acid sequences.

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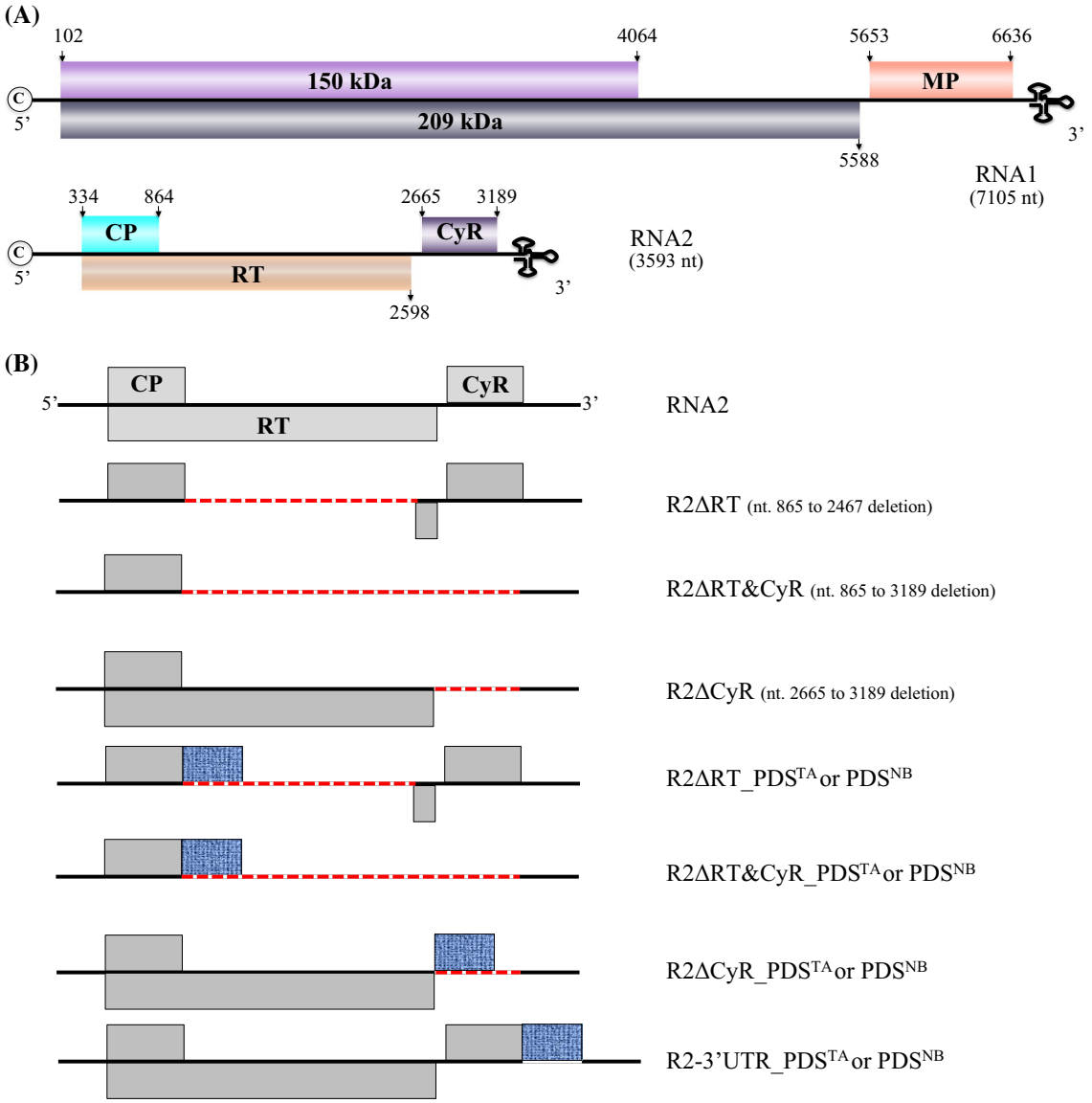

Fig. 1 Schematic representation of SBWMV genome and mutant clones. (A) RNA1 and RNA2 genome maps are shown, indicating the 5' cap, the 3' tRNA like structure, and the coding regions in boxes with nucleotide coordinates. (B) RNA2-derived cDNA clones with deletions or insertions. Deletions in the wild-type virus sequence are shown with red dashed lines. PDS sequence insertions from *N. benthamiana* (370 nt) or *T. aestivum* (409 nt) in the antisense orientation are shown as blue textured boxes (color figure online)

Full-length cDNA copies of SBWMV RNAs were cloned downstream of an enhanced cauliflower mosaic virus 35S promoter by replacing the 35S:hygromycin cassette in the original pCAMBIA1380 vector (AF234301.1) [11]. A 49-nt hammerhead ribozyme sequence derived from subterranean clover mottle virus satellite RNA (5'-GTCTGTACTTATATCAGTACACTGACGAGTCCCTAAAGGACGAAACGGG-3') was inserted at the 3' end of viral sequence to ensure proper transcriptional termination [5, 11]. Gibson assembly [9] was used for generating the different cDNA clones. *E. coli* (JM109; Promega) transformations of full-length RNA1-specific clones produced no colonies when maintained at 37 °C, but colonies grew on plates maintained at 28 °C after about 36 hours. Liquid cultures of RNA1 clones were also grown at 28 °C. cDNA clones derived from full-length RNA2 did not show any temperature-related effects. The infectivity of the cDNA clones was tested in *N. benthamiana* through agro-coinfiltrations by mixing equal volumes of agrocultures (*Agrobacterium tumefaciens* strain C58C1; $OD_{600} = 0.8$) containing plasmids with SBWMV RNA1, RNA2 and a 35S-driven tomato bushy stunt virus P19 gene construct. P19, a well-characterized suppressor of RNA silencing that sequesters small interfering (si)

RNAs, was used in all agro-coinfiltration experiments [16]. The replication and encapsidation competency of SBWMV cDNA clones were verified by Northern blot analysis and immunosorbent electron microscopy assays (data not shown). The upper non-inoculated leaves of agro-infiltrated *N. benthamiana* plants showed severe mosaic and leaf deformation symptoms approximately 14-20 days post-infiltration (dpi). Both RNAs were essential for producing symptoms in *N. benthamiana* and wheat, as was reported earlier in wheat and *Chenopodium quinoa* [13, 18]. Infiltrated leaf tissue extracts of *N. benthamiana* at 5-7 dpi were used as scaled up and encapsidated inoculum for transmission of the virus to wheat (line OH05-200-74, kindly provided by Dr. Eric Stockinger, Ohio State University) through mechanical rub-inoculations. At approximately 12 days post-rub-inoculation (dpr), newly emerging noninoculated wheat leaves showed virus symptoms. The results demonstrate the infectivity of our agro-based cDNA clone in *N. benthamiana* and wheat (Fig. 2A and B).

The bipartite rod-shaped morphology and taxonomic proximity of SBWMV to other viruses commonly used as heterologous gene expression or silencing vectors, such as tobacco rattle virus (TRV) and barley stripe mosaic virus (BSMV), prompted our interest in investigating its efficacy as a viral vector for monocotyledonous plants. Due to many challenges associated with viruses infecting monocotyledonous plants, very few are adapted for virus-induced gene silencing (VIGS). Barley stripe mosaic virus (BSMV) and brome mosaic virus (BMV) are relatively well characterized and successfully adapted for low-throughput virus vector applications, with BSMV useful in wheat, barley, oats and *Brachypodium*, and BMV can be used in maize, barley, rice and sorghum [6, 7, 15, 17]. We evaluated SBWMV as a viral vector for gene silencing and gene expression by insertion of green fluorescent protein (GFP) or plant phytoene desaturase (PDS) coding sequences at multiple sites in the viral RNA2 (Fig. 1B and 3A)

The occurrence of spontaneous deletions in the read-through region of CPRT during prolonged infection or mechanical virus transmission yielding fully infectious, mechanically transmissible virus was previously reported in several studies on furoviruses [3, 18, 22]. Except for its putative role in viral transmission through soil-borne zoospores, CPRT is not essential for any vital biological functions such as virus replication, encapsidation and systemic infection [14, 21, 22], making the CPRT region an obvious choice for sequence insertions. Our R2ΔRT RNA2 deletion mutant lacking 1603 nt of the 1734-nt CPRT (R2ΔRT; deletion at nt 865-2467) was able to replicate and systemically infect *N. benthamiana* (Fig. 2A) and wheat (Fig. 2B). To test the utility of the CPRT region as an insertion site, two different PDS insertion clones were developed by replacing the 1603-nt CPRT fragment with insertion sequences. Partial PDS gene sequences derived from *N. benthamiana* (370 bp; nt 21-390 of AJ571700) and *T. aestivum* (409 bp; nt 1025-1433 of FJ517553) were inserted in the antisense orientation. Both of the CPRT site PDS insertion clones, R2ΔRT_PDS^{NB} and R2ΔRT_PDS^{TA}, caused systemic infection and induced visible photobleaching in the non-inoculated leaves of *N. benthamiana* (Fig. 2A). Photobleaching could still be observed on the fresh flush of *N. benthamiana* leaves five months after infiltration (161 dpi) with R2ΔRT_PDS^{NB} (Fig. 2A). R2ΔRT_PDS^{NB}- and R2ΔRT_PDS^{TA}-infiltrated leaf extracts showed systemic infection and symptoms when rub-inoculated to wheat. However, no PDS gene silencing phenotype was observed in wheat from either clone (Fig. 2B). In contrast, a barley stripe mosaic virus vector (pCa-γb:*TaPDS*400, kindly gifted by Dr. Dawei Li) [23] carrying a similar PDS insert sequence (nt 1029-1428 of FJ517553; 400 nt in the sense orientation) resulted in strong PDS silencing in wheat at 7-10 dpr (Fig. 2C). The 409-nt wheat PDS used in this study (nt 1025-1433 of FJ517553) is the same sequence plus nine additional nucleotides, inserted in the antisense orientation, suggesting that the target sequence selection is likely not limiting VIGS in wheat.

CPRT site PDS insertion clones were evaluated for insert stability using primers flanking the insertion site, SP203 (nt 1-27 of KT736089) and SP188 (nt 3224-3247 of KT736089), 837 and 757 nt apart from the insertion site, respectively. R2ΔRT_PDS^{TA}-inoculated *N. benthamiana* leaf samples were collected at 9 dpi from infiltrated leaves, and at 27, 35, 55, 71 dpi from systemically infected leaves. Systemically infected leaves from the corresponding rub-inoculated wheat plants were analyzed at 11, 19, 27, 47 and 63 dpr. A smear of products was observed from *N. benthamiana* samples (Fig. 2D i). Samples collected from wheat showed a stable insert up to 47 dpr (Fig. 2D ii). A similar insert stability analysis was conducted with R2ΔRT_PDS^{NB}-inoculated plants, with leaf samples collected from *N. benthamiana* at 1, 3, 5, 7 dpi (infiltrated leaves) and 14, 25, 55 dpi (systemically infected leaves) and from systemically infected wheat leaves at 13, 18, 31, 49 dpr (Fig. 2D iii). The results indicate that the inserted PDS sequence is relatively stable in infected wheat, despite the lack of a silencing phenotype. RT-PCR analysis with the wild-type virus clone yielded similar results (data not shown).

To test the utility of other sites for sequence insertions, additional clones were developed, replacing the CyR coding region alone (i.e., R2ΔCyR) or in combination with CPRT deletion (i.e., R2ΔRT&CyR). PDS sequence insertions in these sites and also between the CyR stop codon

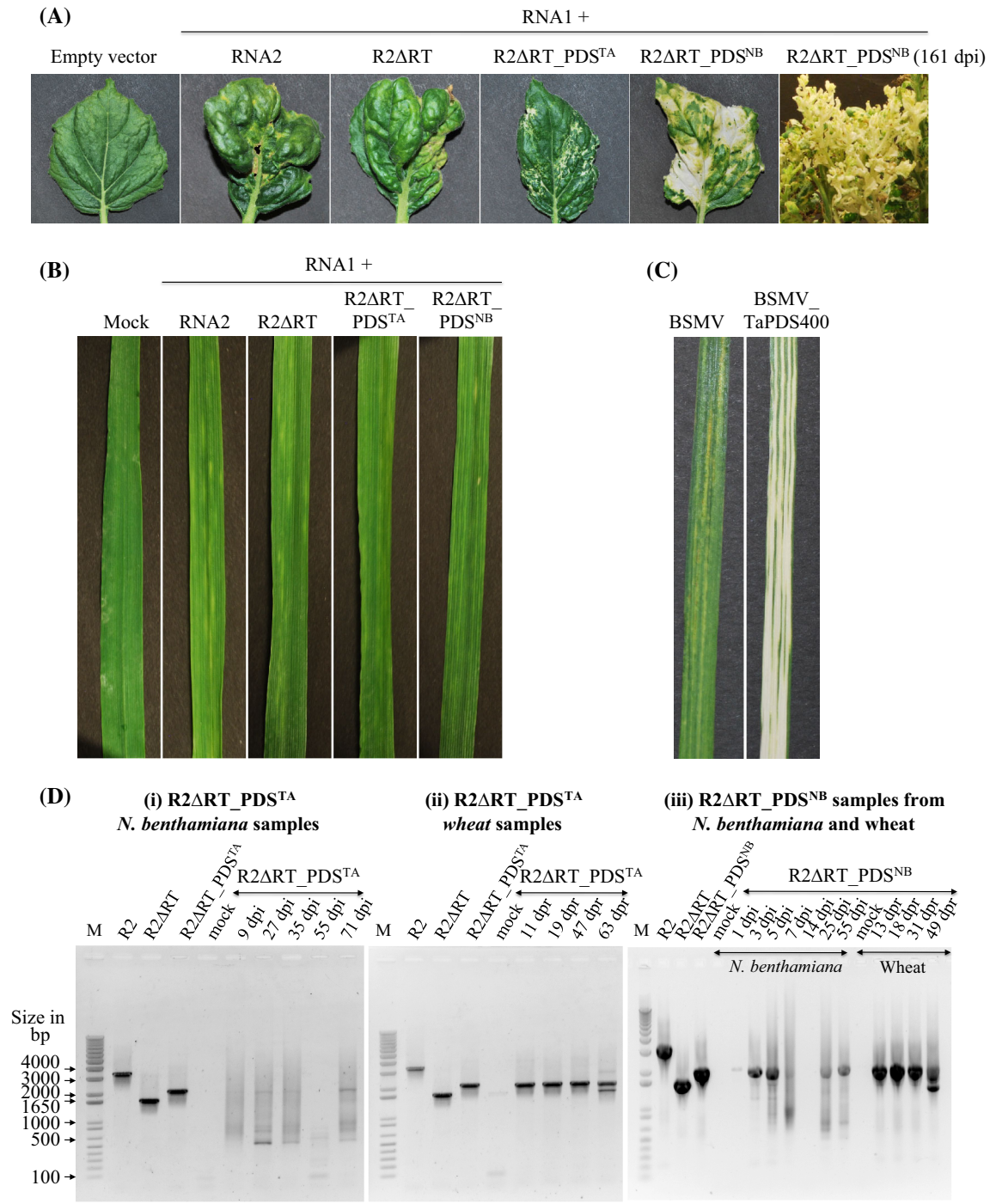

Fig. 2 Assessment of SBWMV as a virus-induced gene silencing vector. (A) Systemic leaves of *N. benthamiana* at 55 dpi, inoculated with RNA1 and the labeled RNA2 cDNA clone. The PDS silencing phenotype observed in the fresh flush of 161 dpi R2ΔRT_PDS^{NB} is also shown. (B) Systemic leaf symptoms observed in wheat at 16 dpr with the same set cDNA clones. (C) Systemic leaves of wheat at 15 dpr with BSMV and BSMV_PDS^{TA400}, respectively, showing wild-type disease symptoms and the PDS silencing phenotype. (D) RT-PCR analysis of PDS insertion clones in CPRT for insert stability. R2ΔRT_PDS^{NB} samples were collected at 9 dpi (infiltrated leaf) and 27, 35, and 55 dpi (systemic leaves) from *N. benthamiana* (i) and at 11, 19, 47 and 63 dpr symptomatic systemic leaves of wheat (ii). For R2ΔRT_PDS^{TA} assay samples were collected at 1, 3, 5, 7 dpi (infiltrated leaf) and 14, 25, 55, 71 dpi (systemic leaves) from *N. benthamiana* and at 13, 18, 31 and 49 dpr from symptomatic systemic leaves of wheat (iii). RT-PCR products were separated on a 1 % agarose gel with a 1 Kb Plus DNA Ladder marker (Invitrogen). Control amplifications from RNA2, R2ΔRT_PDS^{TA} and R2ΔRT cDNA clone templates and mock-inoculated sample RNAs are shown in each panel

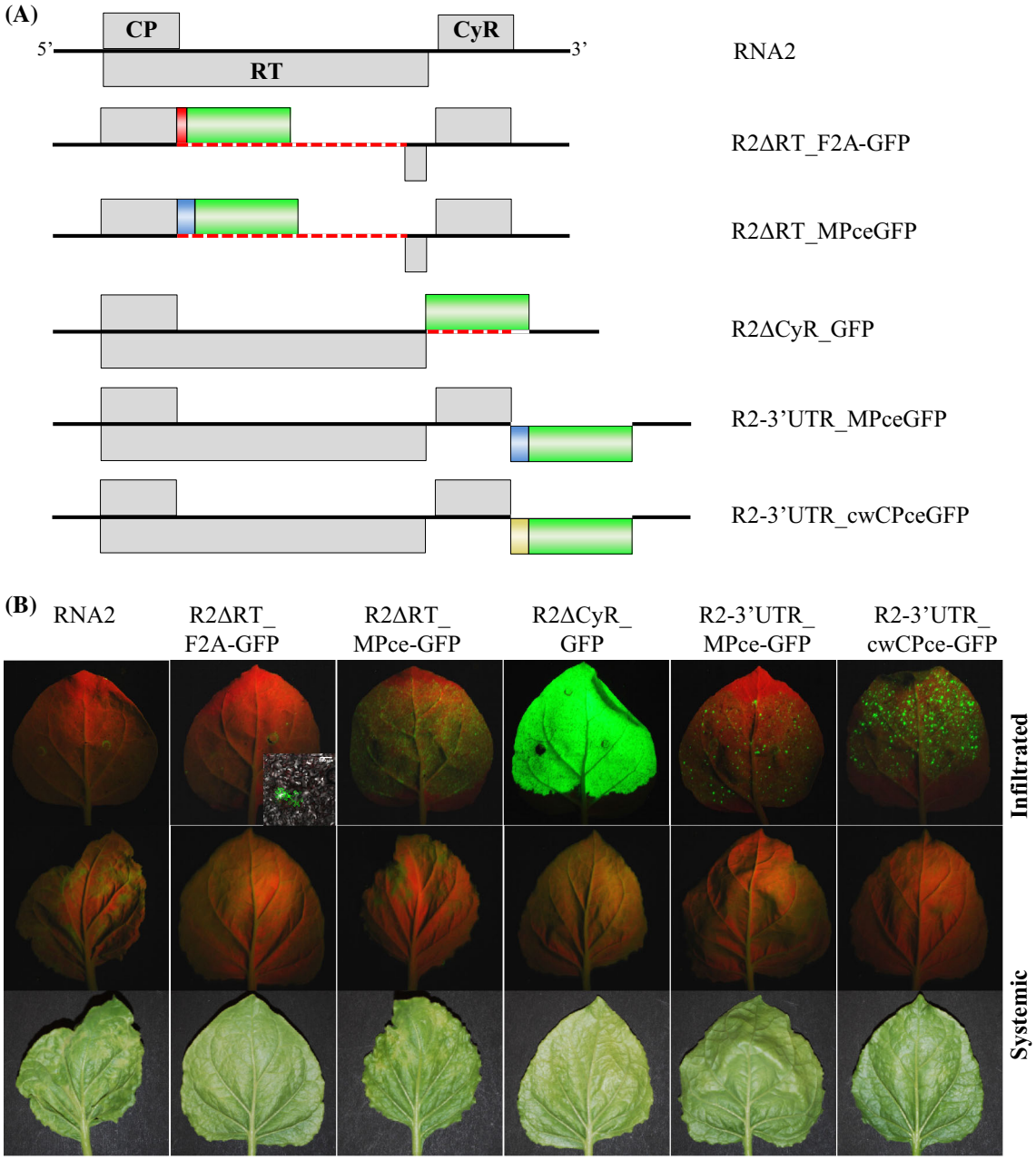

Fig. 3 Assessment of SBWMV as a gene expression vector. (A) Schematic representation of RNA2-derived cDNA clones with GFP (720-nt) gene insertions. GFP gene insertions are shown as green boxes, and upstream F2A protease, MPce and CWMV CPce sequences are shown, respectively, as red, blue, and yellow boxes. (B) Infiltrated (6 dpi; row 1) and systemic (65 dpi; rows 2 and 3) leaves of *N. benthamiana* inoculated with RNA1 and the indicated RNA2 cDNA clone. Images were taken under UV (rows 1 and 2) or transmitted light (row 3) to observe GFP expression and virus infection. Inset: single-cell fluorescence observed in infiltrated leaves of R2ΔRT_F2A-GFP-inoculated *N. benthamiana* through confocal microscopic imaging (color figure online)

and the 3' untranslated region (3'UTR) were assayed (Fig. 1B). None of these clones lacking CyR (R2ΔRT& CyR, R2ΔRT&CyR_PDS^{NB}, R2ΔRT&CyR_PDS^{TA}, R2ΔCyR, R2ΔCyR_PDS^{NB}, and R2ΔCyR_PDS^{TA}) or insertions downstream of CyR (R2-3'UTR_PDS^{NB}, R2-3'UTR_PDS^{TA}) showed systemic infection or photobleaching in either *N. benthamiana* or *T. aestivum*. The loss of systemic infection of CyR deletion clones supports the essentiality of the CyR, which has been reported to be a viral suppressor of host RNA silencing [4]. Loss of infectivity of 3' UTR insertion constructs suggests that the 3' UTR sequence configuration may also be essential.

In addition to VIGS capacity, the SBWMV clone was tested for gene expression capacity using GFP. CPRT and

CyR insertion sites were evaluated for gene expression by inserting a 720-nt cycle 3 GFP [4] coding sequence. Five GFP gene insertion clones were developed: R2ΔRT_F2A-GFP, R2ΔRT_MPceGFP, R2ΔCyR_GFP, R2-3'UTR_MPceGFP and R2-3'UTR_cwCPceGFP (Fig. 3A). In the R2ΔRT_F2A-GFP clone, GFP was fused to the C-terminus of CP by replacing the stop codon with the 24-amino-acid F2A protease sequence for expression as a cleaved fusion protein [10]. Constructs with GFP under potential controller elements (ce) of homologous RNA1-MP or the heterologous Chinese wheat mosaic virus RNA2-CP were built using 125 nt upstream sequence of the indicated open reading frame, since the CyR sgRNA controller sequences of JSBWMV are mapped to 125 nt upstream sequence of the CyR start codon [22]. All five GFP clones showed GFP expression in agroinfiltrated leaves of *N. benthamiana*. The R2ΔCyR_GFP clone in which the CyR coding sequence was replaced with GFP showed the strongest fluorescence of the five constructs. The R2ΔRT_MPceGFP, R2-3'UTR_MPceGFP and R2-3'UTR_cwCPceGFP clones showed moderate GFP gene expression as foci of multiple cells in inoculated leaves. GFP expression from R2ΔRT_F2A-GFP was predominantly restricted to single cells (Fig. 3B). Systemic infection in *N. benthamiana* was observed only with R2ΔRT_MPceGFP, and no GFP expression was observed in the systemic leaves of this or any other construct. Rub inoculations of infiltrated leaf extracts into wheat did not result in symptomatic infection or GFP expression from any of the GFP clones.

The agro-based cDNA clone system developed for the OH isolate of SBWMV is a valuable addition to the currently available reverse genetic tools for this virus. Our results with SBWMV clones with insertions in CPRT showed the ability to induce silencing in the experimental dicotyledonous host *N. benthamiana*, but not in the natural monocotyledonous host *T. aestivum,* despite systemic movement and the remarkable stability of 370- to 409-nt PDS inserts. Insert stability is one of the problems associated with the currently available gene silencing vectors such as BSMV and BMV that are commonly used as gene silencing vectors in monocotyledonous plants [1, 2, 23]. This study indicates the importance and necessity of basic science research to understand potential differences in monocotyledonous virus hosts or specific host-virus combinations.

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References

1. Benavente LM, Ding XS, Redinbaugh MG, Nelson RS, Balint-Kurti P (2012) Virus-induced gene silencing in diverse maize lines using the brome mosaic virus-based silencing vector. Maydica 57:206–214
2. 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 20:1323–1331
3. Chen JP, Macfarlane SA, Wilson TMA (1995) An analysis of spontaneous deletion sites in soil-borne wheat mosaic-virus Rna2. Virology 209:213–217
4. Crameri A, Whitehorn EA, Tate E, Stemmer WP (1996) Improved green fluorescent protein by molecular evolution using DNA shuffling. Nat Biotechnol 14:315–319
5. Davies C, Haseloff J, Symons RH (1990) Structure, self-cleavage, and replication of two viroid-like satellite RNAs (virusoids) of subterranean clover mottle virus. Virology 177:216–224
6. Ding XS, Schneider WL, Chaluvadi SR, Mian MAR, 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 19:1229–1239
7. Ding XS, Rao CS, Nelson RS (2007) Analysis of gene function in rice through virus-induced gene silencing. Methods Mol Biol 354:145–160
8. Estes AP, Brakke MK (1966) Correlation of *Polymyxa graminis* with transmission of soil-borne wheat mosaic virus. Virology 28:772–774
9. Gibson DG, Young L, Chuang R-Y, Venter JC, Hutchison CA, Smith HO (2009) Enzymatic assembly of DNA molecules up to several hundred kilobases. Nat Methods 6:343–345
10. Gopinath K, Wellink J, Porta C, Taylor KM, Lomonossoff GP, van Kammen A (2000) Engineering cowpea mosaic virus RNA-2 into a vector to express heterologous proteins in plants. Virology 267:159–173
11. Gowda S, Satyanarayana T, Robertson C, Garnsey S, Dawson W (2005) Infection of citrus plants with virions generated in *Nicotiana benthamiana* plants agroinfiltrated with a binary vector based *Citrus tristeza* virus. In: Proceedings of the 16th conference of the international organization of citrus virologists. IOCV, Riverside, pp 23–33
12. Kuhne T (2009) Soil-borne viruses affecting cereals—known for long but still a threat. Virus Res 141:174–183
13. Miyanishi M, Roh SH, Yamamiya A, Ohsato S, Shirako Y (2002) Reassortment between genetically distinct Japanese and US strains of Soil-borne wheat mosaic virus: RNA1 from a Japanese strain and RNA2 from a US strain make a pseudorecombinant virus. Arch Virol 147:1141–1153
14. Ohsato S, Miyanishi M, Shirako Y (2003) The optimal temperature for RNA replication in cells infected by soil-borne wheat mosaic virus is 17 °C. J Gen Virol 84:995–1000
15. Pacak A, Geisler K, Jorgensen B, Barciszewska-Pacak M, Nilsson L, Nielsen TH, 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
16. Scholthof HB (2006) The Tombusvirus-encoded P19: from irrelevance to elegance. Nat Rev Microbiol 4:405–411
17. 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 138:2165–2173
18. Shirako Y, Brakke MK (1984) 2 purified RNAs of soil-borne wheat mosaic-virus are needed for infection. J Gen Virol 65:119–127

19. Shirako Y, Wilson TMA (1993) Complete nucleotide-sequence and organization of the bipartite RNA genome of soil-borne wheat mosaic-virus. Virology 195:16–32
20. Stewart LR, Paul PA, Qu F, Redinbaugh MG, Miao H, Todd J, Jones M (2013) Wheat mosaic virus (WMoV), the causal agent of high plains disease, is present in Ohio wheat fields. Plant Dis 97:1125
21. Yamamiya A, Shirako Y (2000) Construction of full-length cDNA clones to Soil-borne wheat mosaic virus RNA1 and RNA2, from which infectious RNAs are transcribed in vitro: virion formation and systemic infection without expression of the N-terminal and C-terminal extensions to the capsid protein. Virology 277:66–75
22. Yamamiya A, Miyanishi M, Shirako Y (2005) Stable deletions arising in the readthrough region of Soil-borne wheat mosaic virus RNA2 define the 5′ limit of the functional promoter for the p19 subgenomic RNA. Arch Virol 150:1871–1884
23. Yuan C, Li C, Yan L, Jackson AO, Liu Z, Han C, Yu J, Li D (2011) A high throughput barley stripe mosaic virus vector for virus induced gene silencing in monocots and dicots. PLoS One 6:e26468