Methods

Rescue of a plant cytorhabdovirus as versatile expression platforms for planthopper and cereal genomic studies

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Summary

- Plant viruses have been used as rapid and cost-effective expression vectors for heterologous protein expression in genomic studies. However, delivering large or multiple foreign proteins in monocots and insect pests is challenging.

- Here, we recovered a recombinant plant cytorhabdovirus, Barley yellow striate mosaic virus (BYSMV), for use as a versatile expression platform in cereals and the small brown planthopper (SBPH, Laodelphax striatellus) insect vector.

- We engineered BYSMV vectors to provide versatile expression platforms for simultaneous expression of three foreign proteins in barley plants and SBPHs. Moreover, BYSMV vectors could express the c. 600-amino-acid β-glucuronidase (GUS) protein and a red fluorescent protein stably in systemically infected leaves and roots of cereals, including wheat, barley, foxtail millet, and maize plants. Moreover, we have demonstrated that BYSMV vectors can be used in barley to analyze biological functions of gibberellic acid (GA) biosynthesis genes. In a major technical advance, BYSMV vectors were developed for simultaneous delivery of CRISPR/Cas9 nuclease and single guide RNAs for genomic editing in Nicotiana benthamiana leaves.

- Taken together, our results provide considerable potential for rapid screening of functional proteins in cereals and planthoppers, and an efficient approach for developing other insect-transmitted negative-strand RNA viruses.

Introduction

Virus-based expression vectors have been successfully developed from plant viruses for numerous high-throughput genomic studies and commercial applications. These vectors provide useful approaches for transient expression of heterologous proteins in genomic studies and for large-scale production of important proteins, such as antibodies and vaccine antigens (Gleba et al., 2007; Peyret & Lomonossoff, 2015). More interestingly, plant virus-based vectors are also efficient tools of delivery of genetically engineered (GE) reagents, such as CRISPR-Cas9 nucleases and single guide RNAs (sgRNAs) to create traits of interest (Zaidi & Mansoor, 2017). Because plant virus vectors can be engineered in weeks with simple preparation procedures and low costs, the technology has advantages over stable transformation-based methods that are time-consuming and labor-demanding (Gleba et al., 2007; Peyret & Lomonossoff, 2015; Ding et al., 2017).

Currently, the vast majority of efforts and successes have been achieved in plant virus-based vectors based on positive-strand RNA viruses and a few plant DNA viruses that infect the model dicot species Nicotiana benthamiana (Gleba et al., 2007; Purkayastha & Dasgupta, 2009; Yuan et al., 2011; Lee et al., 2012; Peyret & Lomonossoff, 2015; Wang et al., 2016; Ding et al., 2017; Zaidi & Mansoor, 2017; Jiang et al., 2018). However, only limited virus vectors are available for economically important monocot crop applications. Moreover, positive-strand RNA viruses and DNA viruses are not suitable for stable expression of large foreign genes because of genetic instabilities and carrying capacity limitations (Matzeit et al., 1991; Shen & Hohn, 1994; Choi et al., 2000; Gleba et al., 2007; Lee et al., 2012; Peyret & Lomonossoff, 2015; Tatineni et al., 2015; Bouton et al., 2018). In contrast to the knowledge about positive-strand RNA virus- and DNA virus-based vectors, expression vectors derived from plant negative-stranded viruses (NSR viruses) have not been extensively studied because of difficulties in engineering infectious cDNA clones (Jackson & Li, 2016).

Classical plant rhabdoviruses are NSR viruses and comprise the Cytorhabdovirus and Nucleorhabdovirus genera based on...
cytoplasmic or nuclear replication and morphogenesis sites (Jackson et al., 2005; Dietzgen et al., 2017). All rhabdovirus genomes encode five conserved structural proteins, including the nucleoprotein (N), the phosphoprotein (P), the matrix protein (M), the glycoprotein (G) and the viral polymerase (L) in a conserved order 3' - N - P - M - G - L - 5'. In addition, rhabdoviruses may encode diverse accessory genes interspersed or overlapping the five conserved genes involved in pathogenesis and/or cell-to-cell movement (Walker et al., 2011). Moreover, the rhabdovirus genomes have an unusual capacity for plasticity as a result of their discontinuous transcription and low rates of genome recombination (Walker et al., 2015). These features are well suited to engineering virus-based expression vectors by inserting foreign genes into well-defined transcription units (Garbutt et al., 2004; Schnell et al., 2005; Pfaller et al., 2015). The first reverse genetics system for a model plant NSR virus, *Sorbus yellow net necroprotophagovirus* (SYNV), has been developed in the model dicot species *Nicotiana benthamiana* (Ganesan et al., 2013; Q. Wang et al., 2015; Jackson & Li, 2016). However, SYNV does not infect monocot plants, so reverse genetic recoveries of monocot plant-infecting rhabdoviruses or other NSR viruses need to be established in order to develop expression vectors for cereal plants.

**Barley yellow striate mosaic virus** (BYSMV), a member of the genus *Cytorhabdovirus*, is transmitted by the small brown planthopper (*SBPH, Laodelphax striatellus*) in a propogative manner (Conti, 1980; Milne & Conti, 1986). BYSMV is transmitted in nature by SBPHs to 26 species of *Gramineae* and affects worldwide crop production (Izadpanah et al., 1991; Makkarouk et al., 1996, 2001, 2004; Di et al., 2014). The BYSMV genome consists of 12 706 nucleotides and encodes 10 proteins in the order 3'-N-P3-P4/P5-P6-M-G-P9-L-5' (Yan et al., 2015). Here, we used BYSMV as a model to develop the first recombinant cytorhabdovirus from cloned cDNAs. We also engineered BYSMV-based vectors as versatile delivery and expression platforms for genomic studies in planthoppers and cereal plants. In addition, the reverse genetic approach developed in the present study provides a template for rescue of other NSR viruses that are transmitted by insect vectors.

### Materials and Methods

**Preparation of host plants and insect vectors**

*Nicotiana benthamiana*, barley (*Hordeum vulgare*), wheat (*Triticum aestivum*), foxtail millet, maize (*Zea mays*) and rice (*Oryza sativa*) plants were cultivated in a climate chamber under 16 h: 8 h, light: dark conditions at 22°C. The SBPHs (*L. striatellus*) were isolated from Hebei province, China, and maintained in the laboratory for nearly 6 yr. BYSMV was isolated from Hebei province, China, and maintained on wheat plants by vector transmission as described previously (Di et al., 2014; Yan et al., 2015). Healthy and viruliferous SBPHs were reared on rice seedlings in an illumination incubator with a controlled environmental climate programmed with 16 h 25°C: 8 h 20°C, light: dark conditions, as described previously (Cao et al., 2018).

**Construction of BYSMV cDNA infectious clones**

*Barley yellow striate mosaic virus*-infected *L. striatellus* were grown with liquid nitrogen, and RNA was extracted from the powder with Trizol reagent as per the manufacturer’s instructions (Invitrogen). To assemble the full-length BYSMV cDNA, two segments (1–6131 and 6132–12 706 nt) of BYSMV cDNA were reverse transcription polymerase chain reaction (RT-PCR)-amplified from BYSMV antigenic RNA with the primers BSY-1/BSY-2 and BSY-3/BSY-4 (Supporting Information Table S1), respectively. The BYSMV1-6131 segment was inserted between the *StuI* and *SalI* sites of pCB301-2 × 35S-Nos (Shen et al., 2014) with an In-Fusion HD Cloning Kit (TaKaRa, Dalian, China). Subsequently, the resulting pCB-BYSMV1-6131 plasmid was digested with *SalI* and *StuI*, and ligated with the BYSMV6131–12 706 segment to produce the pCB-BYSMV plasmid containing the complete BYSMV cDNA. The constructions of pBY-RFP, pBY-GFP, pBY-GR, and pBY-GRC, pBY-GusR, pBY-GA2ox1R, pBY-GA5R, and pBY-B2R plasmid derivatives are described in Methods S1. The pGD vector (Goodin et al., 2002) derivatives, and pGD-NLP and pGD-VSRs plasmids are described in the Methods S1 and in previous studies (Fang et al., 2019). All the primers used in this study were listed in Table S1.

**Agrobacterium infiltration and insect transmission**

*Agrobacterium* infiltration was performed as described previously (X.-P. Zhang et al., 2017). The *Agrobacterium tumefaciens* EHA105 strains harboring the BYSMV infectious clones, and pGD-NLP and pGD-VSRs plasmids were cultivated overnight at 28°C, harvested by centrifugation, and resuspended in infiltration buffer (10 mM MES, 10 mM MgCl₂ and 150 μM acetylserine). After incubation at room temperature for 3 h, bacterial suspensions harboring the BYSMV virus derivatives (OD₆₀₀ = 1.0), pGD-NLP (OD₆₀₀ = 0.5), and pGD-VSRs (OD₆₀₀ = 0.3) were mixed and infiltrated into *N. benthamiana* leaves at the six-leaf stage. At 14–20 d postinfiltration (dpi), inoculated leaves were homogenized in extraction buffer (100 mM Tris-HCl, 10 mM Mg (CH₃COO)₂, 1 mM MnCl₂, and 40 mM Na₂SO₄, pH 8.4) and centrifuged at 12 000 g for 10 min at 4°C (Jackson & Wagner, 1998). Then, 13.8 ml of the crude supernatants were injected into the thoraces of planthoppers as reported previously (Liu et al., 2010). Briefly, second-instar nymphs were collected and anesthetized in ice. Then, the insects were injected with 13.8 ml of crude leaf extract using a Nanoject II auto-nanoliter injector (Drummond Scientific Co., Broomall, PA, USA). The injected nymphs were maintained on fresh rice seedlings for a 10 d incubation period, and then transferred to healthy barley, wheat, and maize plants for a 2 d inoculation period. These plants were subsequently observed at various times for symptom expression or image analyses.

**Western blotting analysis**

Western blotting analysis was performed as described previously (Dong et al., 2016). Briefly, plant tissues were ground with liquid
nitrile and incubated with sodium dodecyl sulfate (SDS) sample buffer (100 mM Tris-HCl, 0.2% bromophenol blue, 20% glycerol, 4% SDS, and 5% β-mercaptoethanol, adjusted pH to 6.8). Total soluble proteins were separated in SDS-polyacrylamide gel electrophoresis gels, transferred to nitrocellulose membranes and tested for the N, P, M, GFP or RFP accumulation with anti-N (1:5000), anti-P (1:3000), anti-M (1:3000), anti-GFP (1:1000), or anti-RFP (1:3000), respectively. Then, the primary antibodies were detected with goat anti-rabbit IgG-conjugated alkaline phosphatase. Anti-G (1:1000), and Anti-his (1:5000) were used to detect accumulation of BYSMV-G protein and 6×His-CFP proteins, then further detected with goat anti-mouse IgG-conjugated alkaline phosphatase. The BYSMV N protein antibody was described previously (Yan et al., 2015; Cao et al., 2018). The BYSMV M and G polyclonal antibodies were prepared from rabbits or mice immunized with prokaryotic-expressed M and a G fragment (amino acids 1–700), respectively. Rubisco bands served as equal loading controls using the Stain-Free technology (Bio-Rad) (Gurtler et al., 2013).

Image acquisition

The fluorescence of plant leaves and insect-dissected tissues was observed with a Zeiss 710 laser scanning confocal microscope. Cyan fluorescent protein (CFP), green fluorescent protein (GFP), and red fluorescent protein (RFP) were excited at 440, 488 and 543 nm wavelength, and emission signals were collected at 450–490, 500–540 and 580–620 nm, respectively. The whole bodies of SBPHs were photographed with an Olympus stereomicroscope SX16 (Olympus, Tokyo, Japan), and GFP, RFP and CFP fluorescence signals were detected with GFP and RFP filters, respectively. To detect accumulation of recombinant BYSMV in SBPHs, the guts and salivary glands were dissected and observed using an Olympus FV1000 microscope.

Gus staining

Plant GUS staining was performed according to a method described previously (Guo et al., 2017). Briefly, the leaves, stems, and roots of BYSMV-infected plants were incubated with X-Gluc staining solution (2 mM potassium ferricyanide, 0.05% Triton X-100, 2 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid, 10 mM phosphate buffer, pH 7.2) in the dark at 37°C for 12 h, and were then dehydrated in an ethanol series. The stained leaves, stems, and roots were viewed and photographed directly. The root tips were photographed using an Olympus SX61 stereomicroscope. GUS staining of the insects was performed according to a modified protocol (Martin-Ortigosa & Wang, 2014). For whole-body staining, beheaded SBPHs were soaked in X-Gluc staining solution and incubated in the dark at 37°C for 12 h. Dissected tissues, guts and salivary glands were pretreated with 0.05% Triton-100 for 30 min and then placed in improved X-Gluc staining solution without Triton reagent for 2 h. The stained tissues were photographed using an Olympus BX53 fluorescence microscope.

BYSMV-mediated genome editing

The Cas9 open reading frame (ORF) was amplified from the pHEC401 plasmid as reported previously (Z. P. Wang et al., 2015). The sgRNA contains a 20 bp guide sequence matching a 20 bp region (226–245 nt) in the GFP gene in N. benthamiana 16c plants (Philips et al., 2017). The sgRNA harboring the gRNA scaffold element was amplified from the pHEC401 plasmid with long primers (Table S1). To construct pBY-Cas9-RFP, the Cas9 ORF and sgRNA were substituted for the GFP and CFP ORFs of pBY-GRC, respectively. The pBY-Cas9-RFP plasmid was transformed into A. tumefaciens and mixed with Agrobacterium harboring the pGD-NLP and pGD-VSRs plasmids, and coinfiltrated into the N. benthamiana 16c line that harbors a GFP transgene for gene editing in these experiments. At 14 dpi, the infiltrated leaves were harvested for genomic DNA extraction using a DNA secure Plant Kit (Tiangen Biotech, Beijing, China). Genomic DNA was digested with NdeI (Takara) for 4 h at 37°C, and used as a PCR template with primers GFP-F and GFP-R (Table S1) using A 2×Phanta Master Mix (Vazyme Biotech Co. Ltd, Nanjing, China). DNAs in the gel bands were purified and cloned with the M5 HiPer pTOPO-Blunt Cloning Kit (Mei5 Biotechnology, Beijing, China) before DNA sequencing.

Electron microscopy

Nicotiana benthamiana and barley tissues containing BYSMV and derivative recombinant virions were excised, fixed and embedded in Spurr’s resin as described previously (Yan et al., 2015). Thin sections were examined with a JEM-1230 transmission electron microscope (Jeol, Tokyo, Japan) to determine virion structure and cytopathology of infected cells.

Statistical analysis

One-way ANOVA with Tukey’s post hoc tests were performed with GRAPHPAD PRISM v.5.00 (GraphPad Software Inc., San Diego, CA, USA) for the comparative evaluation of the height of barley.

Results

Recovery of infectious BYSMV from cloned cDNAs in N. benthamiana leaves

To generate a wild-type BYSMV cDNA clone, two BYSMV cDNA segments (1–6131 nt and 6132–12 706 nt) were separately amplified from BYSMV-infected L. striatellus using RT-PCR, and assembled to create the full-length BYSMV cDNA. Then, the full-length BYSMV cDNA was cloned into pCB301-2 × 35S-Nos between the double cauliflower mosaic virus 35S promoter (2 × 35S) and the hepatitis delta virus ribozyme sequence (Shen et al., 2014).

To monitor virus infection in vivo, we next engineered a BYSMV vector with an RFP gene insertion. To this end, the RFP gene was flanked by the N/P gene junction sequences (Yan et al.,
and this derivative was inserted between the BYSMV N and P genes (Fig. 1a). For NSR viruses, the nucleocapsid core proteins function in viral transcription and replication, and all rhabdoviruses require the N, P and L core proteins for regeneration of infectious cDNA clones in vivo (Jackson & Li, 2016). Thus, the BYSMV N, P and L ORFs were individually inserted into the pGD vector, and then the three expression cassettes were inserted into one plasmid (pGD-NLP) (Fig. 1a). To further enhance accumulations of BYSMV N, P and L proteins in plants, coexpression of viral suppressors of RNA silencing (VSRs) are required to inhibit host RNA silencing responses. Therefore, three VSRs, including the Tomato bushy stunt virus p19, the Tobacco etch virus HC-Pro, and the Barley stripe mosaic virus γb VSR proteins, were individually inserted into the pGD vector, and then three expression cassettes were combined into one plasmid, designated as pGD-VSRs (Fig. 1a).

To rescue the recombinant BY-RFP cDNA constructs, the pBY-RFP, pGD-NLP and pGD-VSR plasmids were transformed into A. tumefaciens and co-infiltrated into N. benthamiana leaves (Fig. 1b). By 14 dpi, high-intensity RFP fluorescence was observed in the cells agroinfiltrated with pBY-RFP, pGD-NLP, and pGD-VSR plasmids (Fig. 1c, right panels). By contrast, RFP fluorescence was not observed in leaves infiltrated with mixtures lacking pGD-NLP (Fig. 1c, left panel). Western blotting analyses revealed that the RFP and BYSMV M proteins had accumulated in the infiltrated N. benthamiana leaves (Fig. S1a). These results indicate that the N, P, and L proteins are functionally active and are able to interact with agRNA transcripts to rescue recombinant pBY-RFP in the agroinfiltrated N. benthamiana leaves.

To confirm the presence of recombinant BYSMV in agroinfiltrated N. benthamiana leaves, leaves were sectioned at 14 dpi and examined using transmission electron microscopy. In contrast to the BYSMV virions (315–353 × 46–57 nm) in the previous study of BYSMV-infected wheat plants (Yan et al., 2015), some smaller bullet-shaped particles of size 200–250 × 18–22 nm (n = 30) were observed in the cytoplasm of an ultrathin section of infected N. benthamiana cells (Fig. S1b) as described previously (Q. Wang et al., 2015). Collectively, these results indicate that the rescued BY-RFP exhibits active virus replication and transcription, and finally forms nonenveloped particles in agroinfiltrated N. benthamiana leaves.

Rescue of recombinant BY-RFP in plant hopper vector and barley plants

Barley yellow striate mosaic virus is transmitted in fields to cereal plants by SBPHs in a persistent-propagative manner, and in a previous study, we maintained BYSMV on cereals by serial SBPH feeding (Cao et al., 2018). However, because N. benthamiana is not a suitable host for the SBPH, it was not possible to transmit BY-RFP from infected N. benthamiana leaves to barley plants using the feeding method described previously (Cao et al., 2018). Therefore, in alternative attempts to obtain viruliferous SBPHs, we ground 2 g of BY-RFP-infected N. benthamiana leaves with 1 ml of extraction buffer at 14 dpi, and injected 13.8 nl of low-speed supernatants into thoraces of healthy SBPHs (Fig. 1b). After injection, the insects were maintained on healthy rice plants during which c. 70% of those injected survived. After a 10 d incubation period, RFP fluorescence was observed in c. 40% of the SPBHs surviving BY-RFP extract injections, but not in mock-injected SBPHs (Fig. 1d). In addition, RFP fluorescence was evident in disected salivary glands and alimentary canals of infected SBPHs (Fig. S2). These results demonstrate that BY-RFP rescued in agroinfiltrated N. benthamiana could be transmitted to SBPHs by injection of leaf extracts.

After a 10 d incubation period on rice plants, the BY-RFP-infected SBPHs were transferred to healthy barley plants (five insects per plant) for a 2 d infection access period. At c. 15 dpi, 90% of the BY-RFP inoculated barley plants developed stunted growth and chlorotic specks on leaves, similar to those observed in wild-type BYSMV infections (Fig. 1e). In addition, the BY-RFP infection exhibited high-intensity RFP fluorescence within a substantial number cells of systemically infected leaves (Fig. 1f). Western blotting showed that abundant RFP accumulated in systemically infected leaves infected by BY-RFP, but not in wild-type BYSMV-infected leaves (Fig. 1g). In addition, accumulations of the P, M, and G viral proteins in the BY-RFP-infected plants were comparable to those of wild-type BYSMV-infected plants (Fig. 1g). Moreover, abundant bacilliform particles of size 315–353 × 46–57 nm were observed in the cytoplasm of thin-sectioned cells infected with either BYSMV or BY-RFP (Fig. 1h). In conclusion, these results demonstrate that the injected SBPHs are viruliferous and able to transmit recombinant BY-RFP to barley plants.

Engineering BYSMV vector platforms for expression of foreign proteins in plants and insects

Given their classical discontinuous transcription and well-defined transcription units, rhabdovirus genomes are able to maintain multiple insertions of transcription units that permit stable foreign protein expression (Jackson et al., 2005). Hence, we attempted to create BYSMV-based vectors for expression of multiple foreign proteins in planta. For this purpose, the GFP gene was first inserted between the N/P gene junctions and the derivative was cloned between the BYSMV N and P genes to produce pBY-GFP (Fig. 2a). Then, pBY-GFP was further manipulated to permit coexpression of the GFP and RFP proteins by inserting the RFP sequence between the GFP and P genes to produce the pBY-GR clone (Fig. 2a). Note that the RFP insertion to produce pBY-GR results in duplication of N/P gene junctions that flank the GFP and RFP genes (Fig. 2a). To further increase simultaneous foreign protein expression, the CFP gene spanned by the P6 and M gene junction and the P/3 gene junction was inserted into pBY-GR between the P and P3 genes to engineer pBY-GR (Fig. 2a).

The pBY-GFP, pBY-GR or pBY-GRC plasmids were expressed in N. benthamiana leaves by agroinfiltration along with the bacterial mixture harboring the pGD-NLP and pGD-VSR plasmids. At c. 14 dpi, crude extracts of the agroinfiltrated N. benthamiana leaves were prepared by grinding leaves in extraction buffer and the low-speed supernatants were injected into thoraces of healthy SBPHs as described earlier (Fig. 1b). After 10 dpi, c. 40% of the injected plant hoppers exhibited GFP, RFP
and/or CFP fluorescence correspondingly (Fig. 2b). Subsequently, these planthoppers were fed on barley plants (five insects per plant) for 2 d inoculation. At c. 15 dpi, GFP fluorescence was observed in c. 90% of the emerging leaves, indicating BY-GFP infections (Fig. 2b, top panels), which could also be monitored under a hand-held UV lamp (Fig. S3). GFP and RFP fluorescence were present within single cells of leaves systemically infected by BY-GR (Fig. 2c, middle panels). Moreover, GFP, RFP, and CFP fluorescence was observed in the cells of systemically infected leaves of BY-GRC-infected barley plants (Fig. 2c, bottom panels).
Engineering BYSMV vectors for expression of large proteins in plants and insects

Next, we assessed whether BYSMV-based vectors could be used for the expression of large foreign proteins. To this end, the GFP ORF of the pBY-GR plasmid was substituted for the gusA gene, which encodes a 600-amino-acid β-glucuronidase (GUS) protein to generate pBY-GusR (Fig. 3a). Note that this substitution was designed to retain use of the downstream RFP reporter gene to monitor systemic infections in plants and SBPHs. Using the strategy described earlier, BY-GusR was rescued in N. benthamiana leaves, and the resulting crude leaf extracts were injected into SBPHs. After a 10 d incubation period on rice plants, the infected SBPHs were transferred to young seedlings of several major cereals, including barley (Hordeum vulgare, cv Golden promise), wheat (Triticum aestivum, cv Yangmai 10), foxtail millet (Setaria italica, Yugu 2), and maize (Zea mays) line B110. After a 2 d inoculation period, the infected SBPHs were observed for RFP expression and histochemically stained for GUS activity. The results showed that high-intensity RFP fluorescence and GUS activity were present in whole bodies, alimentary canals, and salivary glands of infected SBPHs (Fig. S4), indicating that the large GUS and RFP insertion did not affect BYSMV infection of the SBPHs.

At 30 dpi, histochemical staining showed that all systemically infected leaves, stems, and roots of wheat, barley, and foxtail millet plants exhibited high-intensity GUS activity, whereas GUS activity was not detected in the mock-treated plant tissues (Fig. 3b). By contrast, GUS activity was only detected as blue streaks along the major and minor veins of systemically infected maize leaves and stems (Fig. 3c). More interestingly, the secondary roots of infected maize exhibited significantly higher GUS activity than the primary roots (Fig. 3c), indicating that the primary roots might have enhanced resistance to virus infection. In addition, the secondary root tips were free of GUS activity (Fig. 3d), which agrees well with the notion that most plant viruses do not infect shoot and root meristems of host plants (Baulcombe, 2004).

The GUS activity results indicate that BY-GusR infection is variable in maize plants compared with the more uniform and extensive infection in barley plants (Fig. 3b,c). Similarly, RFP fluorescence was also patchy in the BY-GusR-infected maize leaves (Fig. 3d, left panels). Moreover, RFP fluorescence from cross-sections of BY-GusR-infected maize leaves suggests that cell-to-cell movement from the infected phloem is limited (Fig. 3d, left panels). By contrast, RFP fluorescence in the systemically infected barley leaves and cross-sections suggested that the cell-to-cell movement of BY-GusR is more extensive than in maize (Fig. 3d, right panels).

In conclusion, our results demonstrate that BYSMV-based vectors provide efficient expression platforms for multiple and/or large proteins in systemically infected leaves of wheat, barley, foxtail millet, and maize. These features obviously provide a model for future analyses of the functions of multicomponent biological complexes in plants and insects.

Participation of BYSMV delivered CRISPR-Cas9 nucleases and guide RNAs (gRNAs) in N. benthamiana genome editing

Given that recombinant BYSMV vectors could deliver multiple and/or large proteins, we next investigated whether BYSMV
derivatives expressing CRISPR-Cas9 nucleases and sgRNAs can mediate genome editing in plants. For this purpose, we first generated the pBY-Cas9-RFP vector, in which the Cas9 nuclease and a sgRNA were substituted, respectively, for the GFP and CFP genes of pBY-GRC (Fig. 4a). The gRNA contains a guide sequence matching a 20 bp region within the GFP gene in N. benthamiana 16c plants (Fig. 4b) (Philips et al., 2017). In addition, the sgRNA targeting sequence contains an NdeI

Fig. 2 Barley yellow striate mosaic virus (BYSMV) expression of foreign proteins in planthoppers and barley plants. (a) Illustration of the pBY-GFP, pBY-GR and pBY-GRC plasmids. The N/P gene junction contains the 3’ untranslated region of the N mRNA (N 3’ UTR), the intergenic sequence (IS), and the 5’ untranslated region of the P mRNA (P 5’ UTR). The P3 gene junction contains the 3’ untranslated region of the P mRNA (P 3’ UTR), the IS, and the 5’ untranslated region of the P3 mRNA (P3 5’ UTR). (b) Confocal microscopy images of green fluorescent protein (GFP), red fluorescent protein (RFP), and cyan fluorescent protein (CFP) expressed from BY-GFP, BY-GR and BY-GRC infections in the small brown planthoppers (SBPHs). SBPH nymphs were microinjected with crude extracts from mock-inoculated or BY-GFP, BY-GR, or BY-GRC-infected N. benthamiana leaves. At 10 d after injection, the insects were photographed with a fluorescence microscope. Bars, 1 mm. (c) Confocal microscopy images of GFP, RFP and CFP in emerging barley leaves systemically infected by BY-GFP, BY-GR and BY-GRC infections. Bars, 20 μm. (d) Western blot analyses showing accumulation of GFP, RFP, CFP, and the BYSMV N and G proteins in the samples shown in panel C using the RFP, P, M, and G specific antibodies. Mock controls were healthy barley plants. Stain-Free technology (Bio-Rad) stained Rubisco bands served as loading control.
Fig. 3 Expression of β-glucuronidase (GUS) and red fluorescent protein (RFP) from Barley yellow striae mosaic virus (BYSMV) vectors in cereal plants. (a) Diagram of the pBY-GusR vector that was generated by replacing the green fluorescent protein (GFP) gene of the pBY-GR vector with the GUS gene. (b) GUS in BY-GusR-infected barley (Hordeum vulgare cv Golden promise), wheat (Triticum aestivum cv Yangmai 10), and foxtail millet (Setaria italica, Yugu 2). Whole plants and detached leaves and roots were stained by X-Gluc at 30 d postinfiltration (dpi). The photographs show representative individuals from three independent experiments. Healthy plants acted as mock controls. Bars, 2 cm. (c) GUS activity in BY-GusR-infected maize leaves, stems, and roots at 30 dpi. The maize leaves from the bottom to the top were designated L1, L2, L3 and L4. In the root photographs, the primary and secondary roots are indicated by arrowheads and arrows, respectively. Healthy maize plants served as mock controls. Bars: (leaves, stems, and roots), 2 cm; (secondary root tips), 1 mm. (d) Confocal microscopy images of RFP fluorescence in systemically infected leaves and cross-sections of the BY-GusR-infected maize and barley plants at 30 dpi. Samples are representative individuals from three independent experiments. Healthy plants acted as mock controls. Bars, 1 mm.
restriction site that will be abolished by successful genome editing (Fig. 4b).

Agrobacterium tumefaciens strains containing pBY-Cas9-RFP, pGD-NLP and pGD-VSRs vectors were agroinfiltrated into Nicotiana benthamiana leaves. At 14 dpi, RFP foci were observed in infiltrated leaves (Fig. 4c), which indicates that the BY-Cas9-RFP infection occurred. Moreover, Western blotting showed that Cas9-Flag protein accumulated in the infiltrated leaves (Fig. 4d). Nicotiana benthamiana genomic DNA from mock- or BY-Cas9-RFP-infected leaves was extracted and used as templates for GFP amplification with primers flanking the GFP target site (Fig. 4e, upper panel). After NdeI digestion, the mock plant DNA
templates failed to produce PCR bands. However, positive PCR bands were obtained from NdeI-treated DNA templates of two independent leaves infected with BY-Cas9-RFP (Fig. 4e, bottom panel). PCR products from mock plants and the NdeI-resistant DNA samples from BY-Cas9-RFP-infected samples were cloned and each clone was sequenced. Sequence analyses revealed that the BY-Cas9-RFP infection induced various indels that abolished the NdeI restriction site (Fig. 4f). These results demonstrate conclusively that BYSMV-based vectors could simultaneously deliver functional CRISPR-Cas9 nucleases and sgRNA for genomic engineering of N. benthamiana leaves and that the RFP expression can be used to monitor tissue targeting sites.

Application of BYSMV-based expression vectors for functional genomics studies

We next sought to determine whether BYSMV-based expression vectors could be used for functional studies in plants. We chose two well-studied genes functioning in the gibberellic acid (GA) signaling cascade that regulate cell elongation and morphogenesis (Sun, 2008). The GA5 (AT4G25420) gene of Arabidopsis thaliana encodes a 377-amino-acid protein controlling a key step in GA biosynthesis (Xu et al., 1995). Conversely, the A. thaliana GA2ox1 (AT1G78440) gene encodes an enzyme that inactivates GA signaling (Thomas et al., 1999). To generate overexpression of the GA5 and GA2ox1 genes, we generated the pBY-GA2ox1R and pBY-GA5R plasmids by replacing the GFP ORF of pBY-GR with the GA5 or GA2ox1 ORFs, respectively (Fig. 5a). In these vectors, the downstream RFP gene was maintained to monitor systemic infections of the recombinant viruses (Fig. 5a).

The BY-GA2ox1-RFP, BY-GA5-RFP and BY-GR viruses were rescued in barley plants as described earlier. By c. 30 dpi, BY-GA2ox1-RFP-infected barley plants exhibited a dwarf phenotype compared with BY-GR-infected barley plants (Fig. 5b). Conversely, the BY-GA5-RFP-infected barley plants were substantially taller than the BY-GR-infected plants (Fig. 5b). Moreover, we measured the plant culm height from the base of plants to the highest leaf collar on the main culm. The results show that mean heights of mock-, BY-GR-, BY-GA2ox1R- and BY-GA5R-infected plants averaged 11.64, 4.1, 1.45 and 9.79 cm, respectively (Fig. 5c). In the systemically infected leaves, accumulation of the GA2ox1 and GA5 mRNAs was verified by RT-PCR (Fig. 5d). Western blotting analysis showed accumulation of BYSMV N and RFP proteins in the infected barley plants (Fig. 5e). These results clearly indicated that the overexpression of GA2ox1 and GA5 induced dwarf and high phenotypes, respectively, which agrees well with their previously reported functions (Sun, 2008).

We also examined the applicability of BYSMV vectors in planthoppers. For this purpose, we used the Flock house virus (FHV) B2 protein, which is a well-known VSR and functions in efficient infections in wild-type flies by inhibiting RNA interference (RNAi) responses (Li et al., 2002; Wang et al., 2006; Han et al., 2011). To examine the B2 function in planthoppers, the pBY-B2 plasmid was generated by substitution of GFP with the FHV B2 gene (Fig. 5a). After rescue in barley plant leaves (Fig. S5), crude extracts of BY-B2R and BY-GR were injected into SBPHs. By 6 dpi, the BY-B2R-infected SBPHs exhibited obvious RFP fluorescence, whereas only faint RFP fluorescence was evident in BY-GR-infected SBPHs (Fig. 5f). By 10 dpi, the BY-GR-infected SBPHs showed more intense RFP fluorescence, but the fluorescence was substantially weaker than that of the BY-B2R-infected SBPHs (Fig. 5f). Western blotting consistently showed that the RFP and N proteins accumulated to higher levels in BY-B2R-infected SBPHs than in BY-GR-infected SBPHs (Fig. 5g). These results indicate that ectopically expressing B2 could facilitate BYSMV infections in SBPHs, in agreement with previous reports (Li et al., 2002; Wang et al., 2006; Han et al., 2011).

Collectively, these experiments demonstrate that heterologous proteins delivered by BYSMV vectors exhibit expected functions in barley plants and insects. Thus, the BYSMV vectors have great potential for high-throughput genomic studies of both plants and insect.

Discussion

Reverse genetics systems for analyses of vertebrate NSR viruses, including rabies virus and vesicular stomatitis virus, have resulted in enormous advances over the past two decades (Schnitt et al., 1994; Lawson et al., 1995; Whelan et al., 1995; Conzelmann, 2004). Unfortunately, application of these advances to plant NSR viruses has been constrained by a number of factors, including plant cell wall barriers, lack of susceptible plant and insect vector cell cultures suitable for cloning, and the absence of cloned plant T7 polymerase expression systems (Ganesan et al., 2013; Q. Wang et al., 2015; Jackson & Li, 2016). These difficulties were overcome recently by development of agroinfiltration strategies for construction of SYNV minireplicons and rescue of full recombinant SYNV from cDNAs in N. benthamiana plants (Ganesan et al., 2013; Q. Wang et al., 2015; Jackson & Li, 2016). These methods are probably suitable for rescue of many NSR viruses that infect N. benthamiana and other dicot plants (Ganesan et al., 2013; Q. Wang et al., 2015; Jackson & Li, 2016), but their direct application to reverse genetic analyses of NSR viruses in monocot plants is hampered by technical difficulties. The main obstacles are lack of efficient gene expression systems in monocots and the fact that all plant rhabdoviruses infecting monocots are obligately transmitted by insects, and hence are unable to be transmitted from plant to plant by mechanical inoculation techniques (Jackson & Li, 2016). However, very early pioneering studies showed that several plant vector-propagated pathogens were able to establish virulent infections after injection of their insect vectors with leaf extracts (Sinha & Chiykowski, 1967; Sylvester & Richardson, 1969; Black, 1984), and these methods enabled us to infect the SBPHs with leaf extracts harboring recombinant BYSMV derivatives. We anticipate that our methods will have broad utility for other plant viruses that are obligately transmitted by propagative insect vectors.

First, we found that BYSMV could establish cellular infection of N. benthamiana leaves through Agrobacterium-mediated transcription of full-length BYSMV antigenomic RNA and coexpression of the N, P, L core proteins and VSRs (Fig. 1). Given that
Fig. 5 Expression of functional genes using Barley yellow striate mosaic virus (BYSMV) vectors in cereals and planthoppers. (a) Diagrams of the pBY-GA2ox1R, pBY-GA5R and pBY-B2R vectors generated by replacement of the GFP gene in the pBY-GR vector with the Arabidopsis AT1G78440 and AT4G25420 genes, and the Flock house virus B2 gene, respectively. (b) Symptom development of barley plants systemically infected with BY-GR, BY-GA2ox1R, or BY-GA5R at 30 d postinfiltration (dpi). Representative plants are shown in the right panels. Bars, 5 cm. (c) Culm heights of barley plants infected with BY-GR, BY-GA2ox1R, and BY-GA5R at 30 dpi. Culm heights were recorded from the base of plants to the highest leaf collar on the main culm. Error bars represent the SEM of at least 10 plants from three independent experiments. Letters above the bars indicate significant differences (P < 0.05) determined by ANOVA followed by Turkey’s multiple comparison test analyses. (d) Reverse transcription polymerase chain reaction of the accumulation of Arabidopsis AT1G78440 and AT4G25420 cDNA genes. Barley actin genes were amplified as positive controls. Healthy barley plants were used as mock controls. (e) Western blots showing accumulation of the BYSMV N protein and red fluorescent protein (RFP) with the anti-N and anti-RFP polyclonal antibodies, respectively. Rubisco band controls visualized with Stain-Free technology (Bio-Rad). (f) Visualization of RFP fluorescence in mock-infected small brown planthoppers or BY-B2R infections at 2, 6, and 12 dpi. Bars, 1 mm. (g) Western blot detection of BYSMV N and RFP proteins with the anti-N and anti-RFP polyclonal antibody, respectively. Rubisco band controls visualized with Stain-Free technology.
BYSMV transmissions to cereals requires vector transmission, we injected planthoppers with sap from BYSMV-infected *N. benthamiana* leaves and succeeded in transferring infectious virus from the plants to planthopper nymphs. We then transferred the viruliferous vectors to barley plants which developed BYSMV symptoms after a 10 d latent period. To our knowledge, the infectious BYSMV cDNA clones we rescued are the first example of a fully reverse genetics system of plant cytorhabdoviruses. Clearly, the approach provides a useful template for rescue of other plant and insect NSR viruses, and provides a mechanism to explore fundamental interactions of NSR viruses with their plant and insect hosts more fully (Ammar *et al.*, 2008).

Plant viruses have been used as protein expression vectors for biotechnological and medical applications for the past two decades. However, the use of plant virus protein expression vectors in monocots has generally been more difficult than in dicotyledonous plants. Our studies provide a versatile delivery and expression platform for monocot plants based on infectious cDNA clones of BYSMV. Five features of the BYSMV-based delivery system appear to be promising tools for stable expression of foreign genes. First, the discontinuous transcription and well-defined transcription units of BYSMV can be easily manipulated for gene engineering without disrupting virus infectivity. Second, the remarkable genomic plasticity of rhabdoviruses permits insertion of transcription units without discernible negative effects on viral replication and gene expression. Indeed, we successfully inserted three transcription units for simultaneous expression of functional GFP, RFP, and CFP reporter proteins (Fig. 2). Third, expressions of foreign proteins can be regulated by the polar transcription characteristic of rhabdoviruses, in which decreasing amounts of transcription of mRNAs is based on their proximity from the 3′ end of the genomic RNA (e.g., N > P > M > G > L mRNAs). In our experiments, the expression of the upstream GFP gene is the most abundant, compared with the low expression levels of the downstream RFP and CFP insertions (Fig. 2).

Another important feature is that multiprotein expression from a single vector circumvents the virus exclusion phenomena that interfere with single cell expression of polypeptides from multiple single vector circumvents the virus exclusion phenomena that interference with single cell expression of polypeptides from multiple viral replication and gene expression. Indeed, we successfully inserted three transcription units for simultaneous expression of functional GFP, RFP, and gRNA could be simultaneously expressed from three independent transcription units in a single BYSMV vector (Fig. 4), in which RFP acts as an observable marker to visualize edited cells. Nonetheless, BYSMV-based genome edited systems cannot currently be recovered in cereals and planthoppers, as a result of their extremely large genomes. But this limitation of BYSMV and other rhabdovirus-based expression systems will be addressed by insertions of newly identified small CAS9 proteins in future studies.

Finally, and importantly, BYSMV has great potential for use in developing efficient tools for expression of functional genes in planthoppers. Planthoppers are important insect pests of crops and are causing considerable damage to crop production in Southeast Asia. In addition, planthoppers are among the most important vectors that transmit plant viruses (Ammar & Nault, 2002). RNAi techniques are widely used for genetic studies mainly through dsRNA delivery by various methods, including artificial feeding, injections, and ingestion (Kanakala & Ghanim, 2016). However, there is an increasing demand for efficient expression vectors in living insects that can be applied for functional analyses of planthoppers and other insect genes. To our knowledge, the BYSMV vectors are the first virus vectors that are suitable for planthopper functional studies, so our findings should open new doors for both plant virus and insect vector interactions.

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**Author contributions**

X-BW, QG, and W-YX designed the study and interpreted the data. QG, W-YX, TY, X-DF, QC, Z-JZ, and Z-HD performed the experiments. X-BW, QG, and W-YX analyzed the data and drafted the manuscript. X-BW, YW, QG, and W-YX proofread and finalized the manuscript. QG and W-YX contributed equally to this work.

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References


### Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

**Fig. S1** Detection of BY-RFP in agroinfiltrated *N. benthamiana* leaves.

**Fig. S2** Observation of RFP fluorescence in dissected alimentary canals and salivary glands of BY-RFP-infected SBPHs.

**Fig. S3** Observation of GFP fluorescence in the BY-GFP-infected barley plants at c. 15 dpi.

**Fig. S4** RFP fluorescence and GUS activity in BY-GusR-infected SBPHs.

**Fig. S5** Symptoms and molecular detection of BY-B2R-infected barley plants.

**Methods S1** Descriptions of plasmid constructions.

**Table S1** Primers used in this study.

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