

SHORT COMMUNICATION

Developing reverse genetics systems of northern cereal mosaic virus to reveal superinfection exclusion of two cytorhabdoviruses in barley plants

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Abstract

Recently, reverse genetics systems of plant negative-stranded RNA (NSR) viruses have been developed to study virus–host interactions. Nonetheless, genetic rescue of plant NSR viruses in both insect vectors and monocot plants is very limited. Northern cereal mosaic virus (NCMV), a plant cytorhabdovirus, causes severe diseases in cereal plants through transmission by the small brown planthopper (SBPH, *Laodelphax striatellus*) in a propagative manner. In this study, we first developed a minireplicon system of NCMV in *Nicotiana benthamiana* plants, and then recovered a recombinant NCMV virus (rNCMV-RFP), with a red fluorescent protein (RFP) insertion, in SBPHs and barley plants. We further used rNCMV-RFP and green fluorescent protein (GFP)-tagged barley yellow striate mosaic virus (rBYSMV-GFP), a closely related cytorhabdovirus, to study superinfection exclusion, a widely observed phenomenon in dicot plants rarely studied in monocot plants. Interestingly, cellular superinfection exclusion of rBYSMV-GFP and rNCMV-RFP was observed in barley leaves. Our results demonstrate that two insect-transmitted cytorhabdoviruses are enemies rather than friends at the cellular level during coinfections in plants.

KEYWORDS

cytorhabdovirus, monocot plants, NCMV, plant negative-stranded RNA viruses, reverse genetics systems, superinfection exclusion (SIE)

Plant negative-stranded RNA (NSR) viruses cause severe diseases in dicot and monocot plants worldwide. Most plant NSR viruses are heavily reliant on specific insect vectors for long-distance transmission in fields. Plant NSR viruses have caused significant yield losses and reduced quality of economically important crops. The reverse genetics system of plant viruses is a powerful technique for investigating virus biology, virus–host interactions, and biotechnological control of plant disease (Zang et al., 2020). Although reverse genetics

systems of plant positive-strand RNA viruses have been established for more than 30 years, recovery of plant NSR viruses from cDNA clones has only been achieved in recent years (Zang et al., 2020). The first reverse genetic systems of plant NSR viruses, *Sonchus yellow net nucleorhabdovirus* (SYNV), including the minireplicon (MR) system and agroinfiltration of full-length infectious cDNA clones, was established in dicot plants (Ganesan et al., 2013; Wang et al., 2015). We used insect injection and transmission to recover a

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cytorhabdovirus, barley yellow striate mosaic virus (BYSMV), from full-length cDNA clones in insect vectors (small brown planthopper [SBPH], *Laodelphax striatellus*) and monocot plants (barley, *Hordeum vulgare*) (Fang et al., 2019; Gao et al., 2019). Subsequently, tomato spotted wilt virus, an economically important, segmented NSR virus, was rescued in *Nicotiana benthamiana* plants through codon optimization and agroinfiltration (Feng et al., 2020). More recently, rose rosette virus, with a seven-segmented NSR genome, was recovered in *N. benthamiana* plants and roses by airbrush delivery (Verchot et al., 2020). Although reverse genetic systems for these plant NSR viruses have been successfully established, recovery of insect-transmitted NSR viruses in their insect vectors and host plants is still limited and remains to be developed extensively for examining the basis of the complicated virus–host plant–insect vector interactions.

Northern cereal mosaic virus (NCMV), a cytorhabdovirus, causes severe disease in barley and wheat plants, and occurs mainly in Japan, Korea, and China (Lee & Shikata, 1977; Murayama & Lu, 1967; Yang et al., 2018). NCMV contains a negative-stranded RNA genome consisting of 13,222 nucleotides (Tanno et al., 2000). The NCMV genome encodes five structural proteins: nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G), and polymerase (L). In addition, the NCMV genome encodes five accessory proteins: P3, P4, P5, P6, and P9 in the order 3'-N-P-P3-P4-P5-P6-M-G-P9-L-5' (Tanno et al., 2000). NCMV and BYSMV are phylogenetically closely related cytorhabdoviruses with similar genome organization and sequence identity (Yan et al., 2015). In addition, the two cytorhabdoviruses share similar host ranges in the family Poaceae, and both are transmitted by SBPHs (Cao et al., 2018). Recently, we developed an antigenomic minireplicon system of BYSMV (BYSMV-agMR) and studied the intracellular movement of the BYSMV P protein (Fang et al., 2019). We rescued the full-length BYSMV from cDNA clones and developed versatile delivery platforms based on BYSMV vectors in cereal plants (*H. vulgare*) and SBPHs (Gao et al., 2019). We used the reverse genetics system of BYSMV to uncover the functions of host factors such as carbon catabolite repression 4 and casein kinase 1 in cytorhabdovirus infection (Gao et al., 2020; Zhang et al., 2020). Despite increasing progress in plant NSR virus studies recently, a reverse genetics system for NCMV remains to be developed for highlighting the biological features of NCMV, as well as its interaction with other NSR viruses.

Superinfection exclusion (SIE) is a well-known phenomenon in which the first established infections of many viruses prevent the subsequent reinfection by the same or closely related viruses (Lauretì et al., 2020). For human and animal viruses, including hepatitis C virus, West Nile virus, vesicular stomatitis virus, human immunodeficiency virus, herpesviruses, and poxviruses, SIE has been shown to prevent secondary infections in single cells (Kobiler et al., 2010; Liao et al., 2017; Nethe et al., 2005; Ramirez et al., 2010; Schaller et al., 2007; Simon et al., 1990; Tscherne et al., 2007; Zou et al., 2009). SIE has also been shown to occur at a cellular level for many plant positive-stranded viruses, like citrus tristeza virus, tobacco mosaic virus, turnip crinkle virus, plum pox virus, soilborne wheat mosaic virus, and apple latent spherical virus (Bergua et al.,

2014; Dietrich & Maiss, 2003; Folimonova, 2012; Julve et al., 2013; Miyashita & Kishino, 2010; Takahashi et al., 2007; Zhang et al., 2017, 2019). More recently, it was reported that the matrix protein of SYNIV, a plant NSR virus, mediated SIE by regulating virion assembly (Zhou et al., 2019). However, due to the lack of reverse genetic systems, reported research on the phenomenon of SIE between two different cytorhabdoviruses in monocot plants has lagged behind those reported in dicot plants. In this study, we first developed a minireplicon of NCMV and then rescued the full-length NCMV from full-length cDNA in SBPHs and barley plants. Based on the NCMV and BYSMV infectious cDNA clones, recombinant viruses NCMV-RFP and BYSMV-GFP expressing red and green fluorescent proteins, respectively, were used to study SIE of these closely related cytorhabdoviruses in monocot plants.

To develop an antigenomic minireplicon (agMR) of NCMV, we generated a reporter cassette based on the NCMV antigenome through substituting the open reading frames (ORFs) of NCMV N and P with the green fluorescent protein (GFP) and red fluorescent protein (RFP) ORFs, respectively. In addition, the intergenic sequences (ISs) between the NCMV P and P3 genes were inserted between the GFP and RFP ORFs, resulting in the pNCMV-agMR plasmid (Figure 1a). The ORFs of the NCMV N, P, and L genes were individually introduced into the pGD vector for ectopic protein expression, which is essential for initiation of plant rhabdovirus infections (Figure 1b). To suppress host RNA silencing responses and improve accumulation of viral core proteins, three viral RNA silencing suppressor genes (VSRs; tomato bushy stunt virus p19, barley stripe mosaic virus γ b, and tobacco etch virus HC-Pro) were inserted into the pGD vector (Figure 1c) described previously (Fang et al., 2019; Gao et al., 2019).

To rescue the NCMV agMR system, *Agrobacterium* harbouring the plasmids pNCMV agMR, pGD-N, -P, -L, and -VSRs were coinfiltrated into *N. benthamiana* leaves. Six days postinfiltration (dpi), GFP and RFP fluorescence was observed in the infiltrated regions of *N. benthamiana* leaves (Figure 1d). In contrast, GFP and RFP fluorescence was not observed in *N. benthamiana* leaves without expression of any one of N, P, L, agMR, or VSRs (Figure 1d). Consistently, western blotting analyses showed that accumulation of GFP and RFP was only detected in infiltrated leaves expressing all components (Figure 1e). In the agMR system, efficient expression of the reporter genes requires delivery of all the DNA structures into single plant cells. To improve the recovery efficiency of the NCMV agMR, the N, P, and L expression cassettes were inserted into a single plasmid designated pGD-NPL (Figure 1f). As expected, pGD-NPL dramatically increased foci numbers compared with the separate pGD-N, pGD-P, and pGD-L plasmids (Figure 1g). These results demonstrate that we have successfully established an efficient NCMV minireplicon system in *N. benthamiana* leaves.

The successful establishment of the NCMV-agMR system indicates that all the core proteins and VSRs function to assemble infectious virus replication complexes (Figure 1). Accordingly, we next generated a full-length construct of the NCMV antigenome with an RFP reporter gene insertion to monitor virus infection. To this end,

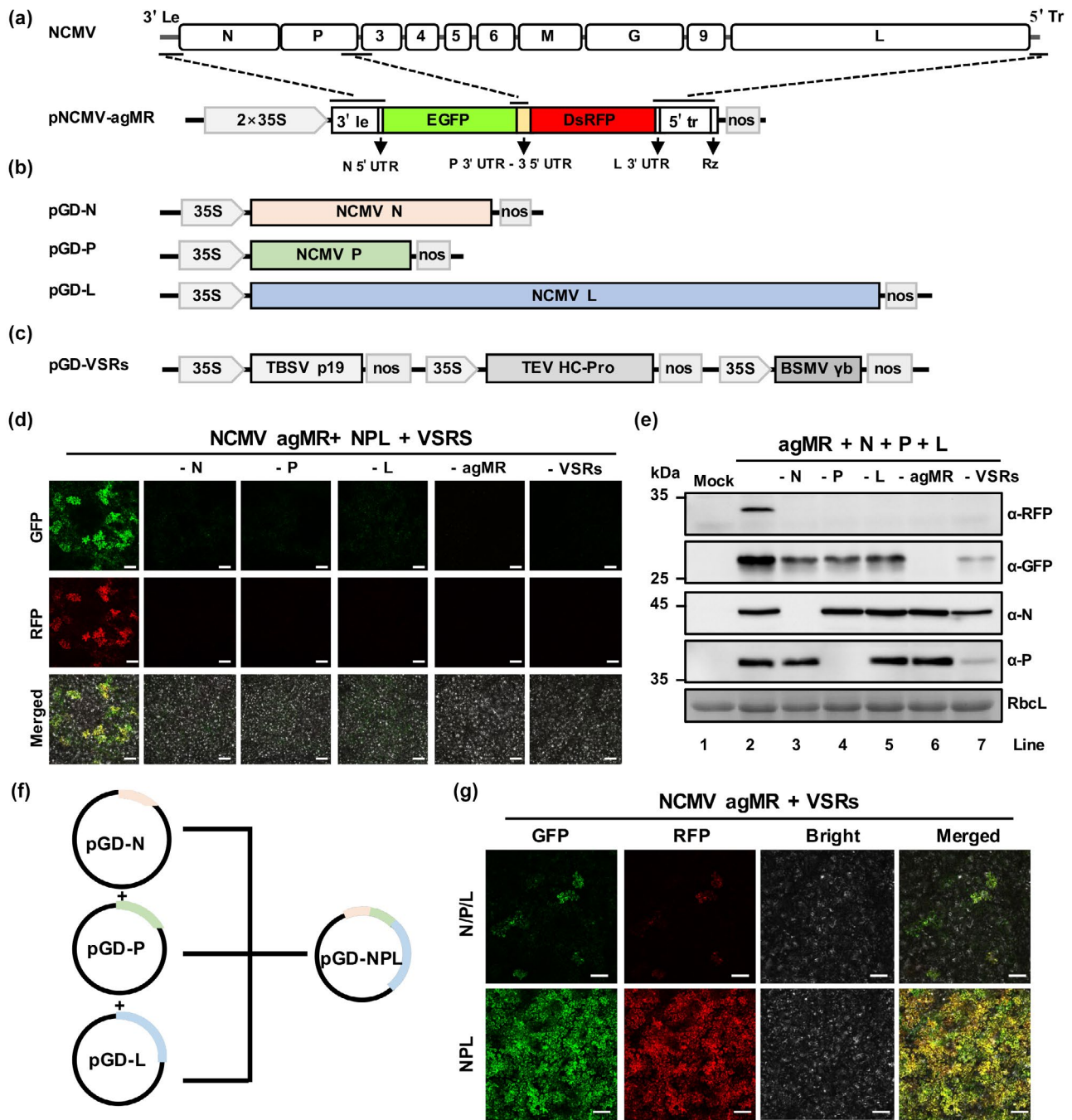


FIGURE 1 Construction of a northern cereal mosaic virus (NCMV) antigenomic minireplicon system in *Nicotiana benthamiana* leaves. (a) Schematic diagram showing a binary construct to express NCMV antigenomic minireplicon. Le, leader. Tr, trailer; UTR, untranslated region; P 3' UTR-3 5' UTR, intergenic sequences including P 3' UTR, intergenic sequence, and 3 5' UTR; Rz, ribozyme sequence; nos, nopaline synthase terminator. (b) Schematic diagrams showing binary constructs to express the NCMV N, P, and L proteins. (c) Schematic diagram showing a binary construct to express the viral RNA silencing suppressor genes (VSRs) tomato bushy stunt virus p19, barley stripe mosaic virus γ B, and tobacco etch virus HC-Pro. (d) green fluorescent protein (GFP) and red fluorescent protein (RFP) foci in *N. benthamiana* infiltrated with different combinations of NCMV agMR system at 6 days postinoculation (dpi) seen with a fluorescence microscope. Scale bars, 200 μ m. (e) Western blot analyses of expression of RFP, GFP, NCMV N and P proteins in leaves shown in (d) with specific antibodies against RFP, GFP, NCMV N and P proteins, respectively. (f) Schematic diagram of representative binary constructs to express NCMV N, P, and L, singly or together. (g) GFP and RFP foci in *N. benthamiana* infiltrated with different combinations of NCMV agMR system at 6 dpi. Scale bars, 200 μ m

the RFP gene flanked by the P/P3 gene junction sequences was amplified and inserted between the NCMV N and P genes (Figure 2a).

To rescue rNCMV-RFP in vivo, *Agrobacterium* harbouring plasmids pNCMV-RFP, pGD-NPL, and pGD-VSRs were coinfiltrated into

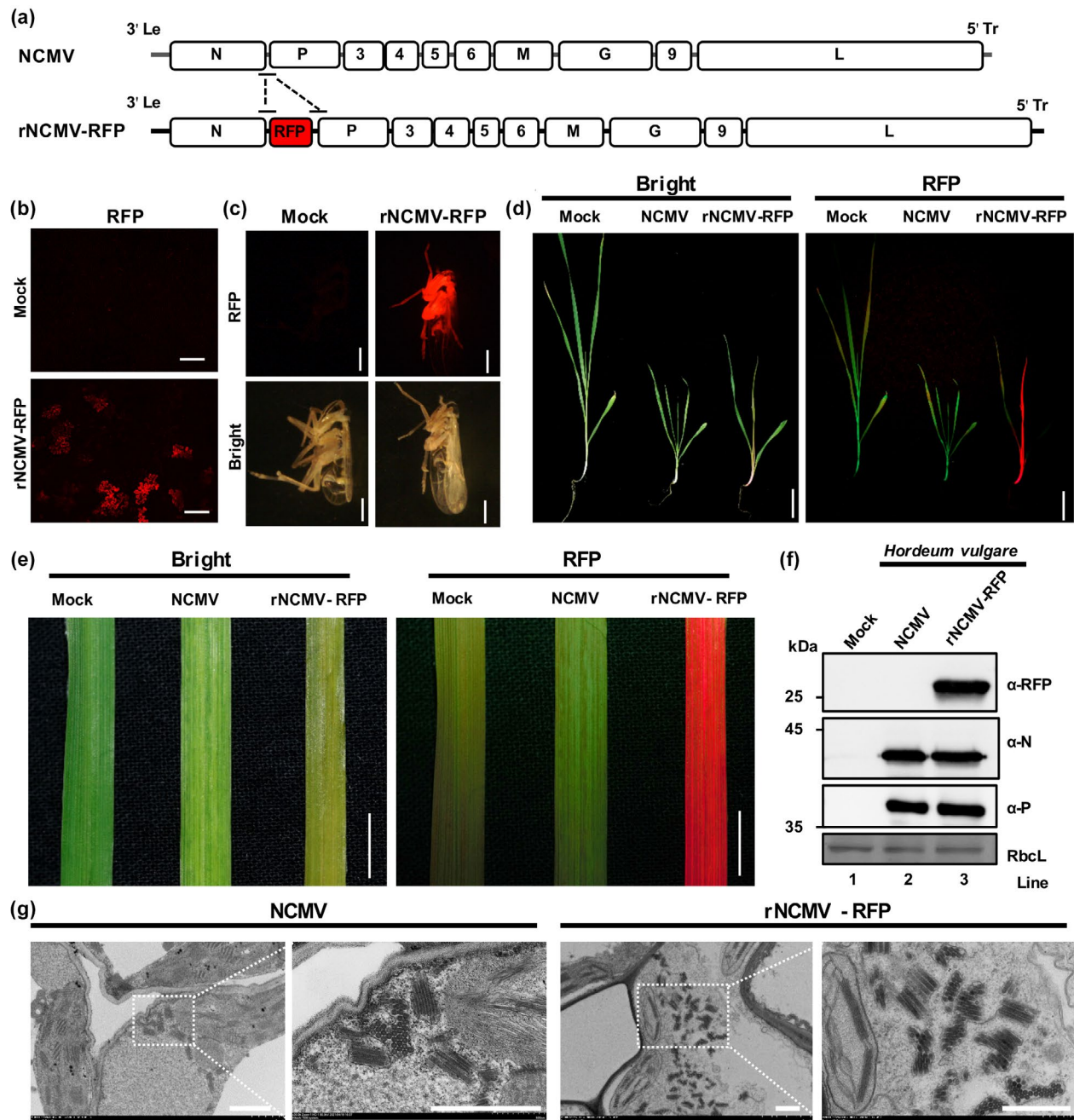


FIGURE 2 Recovery of infectious northern cereal mosaic virus (NCMV) in *Nicotiana benthamiana* leaves, small brown planthoppers (SBPHs, *Laodelphax striatellus*), and barley plants (*Hordeum vulgare*). (a) Schematic diagram of the full-length northern cereal mosaic virus-RFP (NCMV-RFP) complementary DNA (cDNA) containing duplicate P/3 gene junctions flanking the red fluorescent protein (RFP) gene between the N and P of NCMV antigenome cDNA. (b) RFP foci in *N. benthamiana* infiltrated with NCMV-RFP, NCMV nucleoprotein (N), phosphoprotein (P), and the large RNA polymerase (L), and viral RNA silencing suppressors (VSRs) at 12 days postinoculation (dpi) seen with a fluorescence microscope. Scale bars, 200 μ m. (c) Images of SBPHs infected by NCMV-RFP. At 10 dpi, the SBPHs were observed with a fluorescence stereomicroscope. Scale bars, 1 mm. (d) Symptoms on NCMV- and recombinant NCMV virus (rNCMV-RFP)-infected barley plants. Scale bars, 1 cm. (e) RFP fluorescence in barley leaves resulting from systemic infection with NCMV and rNCMV-RFP. Scale bars, 1 cm. (f) Western blot analysis of NCMV proteins and RFP expression in plants shown in (d) with specific antibodies against NCMV N, P, and RFP proteins. (g) Electron micrographs of thin sections of barley leaves infected with NCMV and rNCMV-RFP

N. benthamiana leaves. At 12 dpi, RFP foci were observed in the cytoplasm of infiltrated leaves (Figure 2b), indicating that rNCMV-RFP was recovered in single cells of *N. benthamiana* leaves.

NCMV is transmitted by SBPHs and our previous studies have demonstrated BYSMV can infect SBPHs by injection of insects with an extract of virus-infected plant leaves (Gao et al., 2019; Qiao et al.,

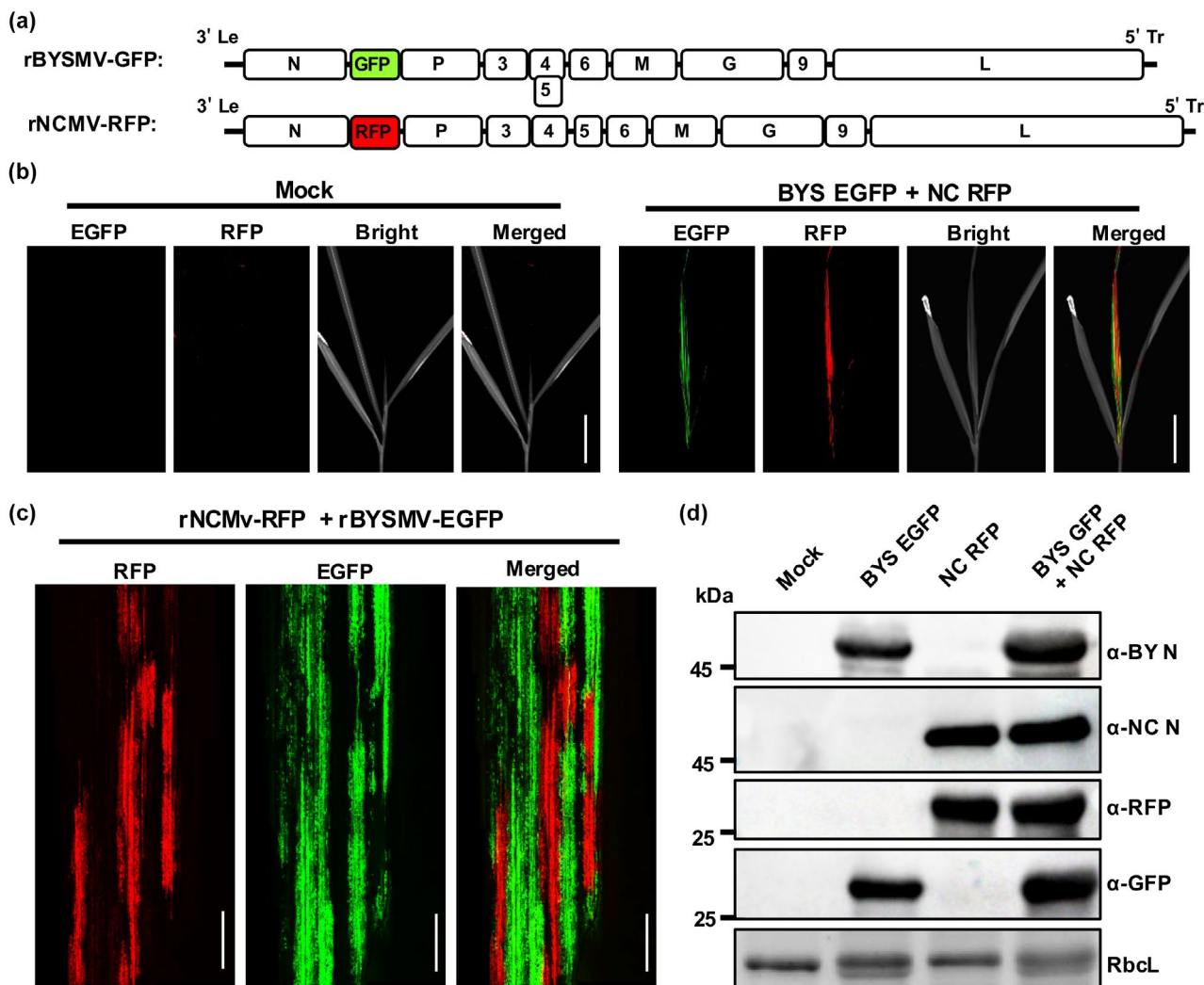


FIGURE 3 Superinfection exclusion (SIE) of recombinant northern cereal mosaic virus (rNCMV-RFP) and GFP-tagged barley yellow striate mosaic virus (rBYSMV-GFP) in barley plants. (a) Schematic diagram of the genome of rNCMV-RFP and rBYSMV-GFP. (b) Coinfection of barley plants by rNCMV-RFP and rBYSMV-GFP at 10 days postinoculation. Scale bars, 3 cm. (c) SIE between rNCMV-RFP and rBYSMV-GFP in barley at the cellular level. Scale bars, 1 μm. (d) Western blot analysis of red fluorescent protein (RFP), green fluorescent protein (GFP), NCMV N, and BYSMV N expression in plants shown in (b) with specific antibodies against RFP, GFP, NCMV N, and BYSMV N proteins, respectively

2022; Tanno et al., 2000). Accordingly, crude extract of rNCMV-RFP-infected *N. benthamiana* leaves was injected into second-instar nymphs and the injected SBPHs were reared on fresh rice seedlings. At 10 dpi, RFP fluorescence was observed in five out of 106 surviving SBPHs (Figure 2c). Immunoblotting analyses showed accumulation of RFP, NCMV N, and P proteins in the injected SBPHs (Figure S1). To confirm that the RFP observed at 10 dpi was due to multiplication of rNCMV-RFP, and not due to the injected extract per se, we examined the SBPHs immediately after injection with crude extract from rNCMV-RFP-infected barley plants (Figure S2). As shown in Figure S2b, the RFP fluorescence intensity of the SBPHs observed immediately after injection was much lower than the SBPHs infected with rNCMV-RFP at 10 dpi, indicating that rNCMV-RFP was successfully rescued in its insect vector.

To determine whether rNCMV-RFP could be transmitted from infected SBPHs to barley plants, we transferred rNCMV-RFP- or wild-type NCMV-infected SBPHs to healthy barley plants for a 3-day infection access period. At 10 dpi, barley plants infected by either rNCMV-RFP or wild-type NCMV exhibited dwarf symptoms compared with mock-treated plants (Figure 2d, left panel), while RFP fluorescence was only observed in leaves systemically infected by rNCMV-RFP, rather than those infected by NCMV (Figure 2d,e). Western blotting analysis showed that abundant RFP accumulated in leaves infected systemically by rNCMV-RFP, but not in those of wild-type NCMV-infected plants (Figure 2f) whereas both N and P proteins were detected in all the infected plants (Figure 2f). In addition, a large number of bacilliform particles were observed in the cytoplasm of barley stem and leaf

cells infected by NCMV or rNCMV-RFP (Figure 2g; Figure S3). In conclusion, these results demonstrate that rNCMV-RFP was successfully rescued in barley plants.

The SIE phenomenon, in which a primary viral infection prevents subsequent viral infection by closely related viruses, has been observed in a broad range of bacterial, plant, and animal viruses (Bergua et al., 2014; Tatineni & French, 2016). However, most of the studies have mainly focused on the SIE by the same virus (Bergua et al., 2014; Whitaker-Dowling et al., 1983; Zhang et al., 2017). Studies of SIE between two different viruses are limited. Tatineni and French (2016) showed that two wheat viruses, wheat streak mosaic virus (WSMV) and Triticum mosaic virus (TriMV), could efficiently coinfect the same wheat cell, suggesting that there was no SIE phenomenon between these two divergent viruses. NCMV and BYSMV are two closely related members of the genus *Cytorhabdovirus* with 62% amino acid identity of their polymerase (L) proteins (Yan et al., 2015). The GFP-tagged BYSMV (rBYSMV-GFP) has been rescued in our previous studies (Gao et al., 2019). To test whether these two viruses could coinfect barley plants, rNCMV-RFP and rBYSMV-GFP were used to examine the nature of coinfection by NCMV and BYSMV in a susceptible barley cultivar, Golden Promise (Figure 3a). Five rNCMV-RFP-infected SBPHs and five rBYSMV-GFP-infected SBPHs were transferred to a healthy barley seedling to coinoculate rNCMV-RFP and rBYSMV-GFP. The upper noninoculated leaves were examined for systemic infection by rNCMV-RFP and rBYSMV-GFP at 10 dpi (Figure 3b,c). Barley leaves observed at the whole-organism level indicated the presence of both GFP and RFP in the same barley leaves (Figure 3b). However, cellular-level observation showed that rNCMV-RFP and rBYSMV-GFP could hardly be detected within the same cell (Figure 3c). Western blot analyses revealed that the proteins NCMV N, BYSMV N, GFP, and RFP were expressed in the expected combinations (Figure 3d). We also observed that two BYSMV variants tagged with the two different fluorescent proteins exhibited SIE in the barley leaves (Figure S4). Taken together, these results reveal that NCMV and BYSMV exhibit SIE at the cellular level, although they coinfect one whole barley plant.

Although NCMV has been reported for more than 70 years, studies about NCMV are limited in morphological and molecular functions due to the lack of a reverse genetic system. Here, we first developed an MR system of NCMV in *N. benthamiana* plants (Figure 1), and then recovered a recombinant NCMV virus (rNCMV-RFP) in SBPHs and barley plants (Figure 2). The successful development of an MR and a full-length reverse genetic system has provided a powerful tool to study the replication and pathogenesis of NCMV. Due to the classical discontinuous transcription and well-defined transcription units of rhabdoviruses, rhabdovirus genomes are able to maintain multiple insertions of transcription units (Gao et al., 2019; Jackson et al., 2005; Xu et al., 2022). The full-length reverse genetic system of NCMV permits stable foreign protein expression in barley plants and

SBPHs, and rapid screening of gene functions in host plants and insect vectors.

The data above suggest that NCMV and BYSMV, two closely related NSR plant viruses, exhibit SIE at the cellular level (Figure 3). Therefore, cross-protection could be used to control virus transmission in the field, which means insulating plants from a disease-causing virus by simply preinoculating them with a mild variant of the same virus (Guo et al., 2020). A large number of plant-infecting viruses are transmitted by insect vectors to their host plants and many of the insect vectors transmit several different virus species (Moreno & Lopez-Moya, 2019). For instance, SBPHs are vectors of both NCMV and BYSMV. It would be interesting to determine whether NCMV and BYSMV can coinfect and be transmitted by one single SBPH. It remains to be determined whether the SIE between NCMV and BYSMV in plants shares similar features in insect vectors. Vector transmission is an essential step in the infection cycle of most plant viruses (Whitfield et al., 2015). Thus, clarifying the phenomenon and mechanism of SIE in insect vectors among various plant viruses could be useful to control the spread and epidemiology of the viruses.

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CONFLICT OF INTEREST

The authors declare that they have no competing interests.

AUTHOR CONTRIBUTIONS

X.F. performed the majority of the experiments, assisted by J.Q., Y.Z., Q.G., D. G. Y.Y., X.L., and W.X. X.F. and X.W. analysed the data. X.W. and X.F. wrote the manuscript, assisted by Y.W. All authors discussed the results and commented on the manuscript.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

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