The cap-snatching frequency of a plant bunyavirus from nonsense mRNAs is low but is increased by silencing of UPF1 or SMG7

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Abstract

Bunyaviruses cleave host cellular mRNAs to acquire cap structures for their own mRNAs in a process called cap-snatching. How bunyaviruses interact with cellular mRNA surveillance pathways such as nonsense-mediated decay (NMD) during cap-snatching remains poorly understood, especially in plants. Rice stripe virus (RSV) is a plant bunyavirus threatening rice production in East Asia. Here, with a newly developed system allowing us to present defined mRNAs to RSV in Nicotiana benthamiana, we found that the frequency of RSV to target nonsense mRNAs (nsRNAs) during cap-snatching was much lower than its frequency to target normal mRNAs. The frequency of RSV to target nsRNAs was increased by virus-induced gene silencing of UPF1 or SMG7, each encoding a protein component involved in early steps of NMD (in an rdr6 RNAi background). Coincidently, RSV accumulation was increased in the UPF1- or SMG7-silenced plants. These data indicated that the frequency of RSV to target nsRNAs during cap-snatching is restricted by NMD. By restricting the frequency of RSV to target nsRNAs, NMD may impose a constraint to the overall cap-snatching efficiency of RSV. Besides a deeper understanding for the cap-snatching of RSV, these findings point to a novel role of NMD in plant–bunyavirus interactions.

Keywords
cap-snatching, nonsense-mediated decay, plant-infecting bunyavirus, rice stripe virus, SMG7, UPF1
(NMD) to eliminate nsRNAs (Kervestin & Jacobson, 2012). The subcellular site where each step of NMD takes place remains a matter of debate. However, there is evidence that nsRNAs may be transported to PBs at later stages of NMD (Chang et al., 2007; Kervestin & Jacobson, 2012; Mérai et al., 2013). Besides nsRNAs, NMD regulates many normal transcripts (Drechsel et al., 2013; Lykke-Andersen & Jensen, 2015). For example, it was estimated that NMD affects more than 20% of the transcriptome in Arabidopsis (Raxwal et al., 2020).

Because cap-snatching and NMD may co-occur in PBs, nsRNAs (and other NMD-regulated transcripts) are ideal cap-snatching targets for bunyaviruses. However, the degradation of nsRNAs may be much faster than that of mRNAs transported to PBs via NMD-unrelated pathways. This suggests that the chance for a bunyavirus to target nsRNAs is low. The two contradictory predictions are reconciled in a finding of Mir et al. (2008), which showed that a hantavirus seems to have a mechanism to inhibit later stages of NMD: the nucleocapsid protein (NP) of the hantavirus has a cap-binding activity. By binding to the cap structure of a nsRNA in PBs, it protects the degradation of the nsRNA from the 5′ terminus. With this mechanism, the hantavirus was shown to target a PTC-containing GFP mRNA more than twofold more frequently than a normal GFP mRNA during cap-snatching (Mir et al., 2008). Such a mechanism predicts that deficiencies in early steps of NMD, which reduce nsRNA accumulation in PBs, are detrimental to the hantavirus. This idea was not tested but was supported by a recent report that showed that Arabidopsis with a deficiency in an early step of NMD shows increased resistance to a plant bunyavirus named tomato spotted wilt virus (TSWV; Ma et al., 2019). However, whether TSWV frequently targets nsRNAs during cap-snatching remains unknown.

Rice stripe tenuivirus is a species of genus Tenuivirus in the family Phenuiviridae of the order Bunyavirales (Xu et al., 2021). By infecting plants of the Gramineae family, particularly rice, rice stripe virus (RSV) poses a serious threat to crop production in some countries of East Asia (Falk & Tsai, 1998). Although many aspects of RSV have been studied intensively in the past decade (Xu et al., 2021), our understanding of the cap-snatching of RSV remains poor (Kormelink et al., 2021; Liu et al., 2016, 2018; Yao et al., 2012). We recently showed that RSV can target mRNAs transiently expressed in Nicotiana benthamiana using agro-infiltration (Lin et al., 2020). This provided us with a system to present defined mRNAs to RSV to investigate its cap-snatching in planta. With the availability of this system and the background described above, we decided to investigate (a) whether RSV targets nsRNAs more frequently in comparison to normal mRNAs during cap-snatching; (b) how deficiencies in early steps of NMD influence the cap-snatching of RSV from nsRNAs; and (c) how deficiencies in early steps of NMD influence the infection of RSV.

A competition assay was used to investigate whether RSV targets nsRNAs more frequently in comparison to normal mRNAs. In this assay, Agrobacterium tumefaciens cell cultures carrying the plasmid pCHF3-C12, which expresses a normal green fluorescent protein gene (GFP) mRNA (GFP- n), or pCHF3-C11-PTC, which expresses a mutant GFP mRNA with a PTC at codon 3 (GFP- m), were mixed at a ratio of 1:1 (see File S1 for experimental procedures). The mixture was infiltrated into a leaf of RSV-infected N. benthamiana (Figure 1a). The infiltrated leaf patch was collected at 3 days post-agro-infiltration (dpi) and RSV NP mRNA in the collected leaf patch was deep sequenced with a method briefly indicated in Figure 1b. The NP mRNA sequences are highly heterogenous with respect to their CRLs because RSV targets a great diversity of cellular mRNAs during cap-snatching (Lin et al., 2017; Liu et al., 2018). For simplicity, RSV NP mRNA sequences with their CRLs acquired from GFP- m and GFP- n are called GFP-m-NP and GFP-n-NP, respectively. GFP-m-NP and GFP-n-NP can be distinguished from each other because RSV cleaves GFP-m and GFP-n at C12 and C11 (Figure 1a), respectively, acquiring two CRLs differing in length (Lin et al., 2020).

The assay was done in triplicate. The total number of NP mRNA sequences obtained from each replicate was 80,664, 99,962, and 79,889. The numbers of GFP-m-NP in the three replicates were 319, 148, and 194, whereas those of GFP-n-NP were 677, 466, and 662. Thus, the accumulation of GFP-m-NP relative to that of GFP- n-NP (GFP-m-NP/GFP-n-NP) in each replicate deviated slightly from a mean value of 0.36:1 (Figure 1c). This indicated that GFP- m had been targeted nearly threefold less frequently than GFP- n by the cap-snatching of RSV.

As the CRL donated by GFP- n is one nucleotide longer than that donated by GFP- m, this observation can be explained by a preference of RSV for longer CRLs. A reciprocal experiment was done to rule out this possibility. In this experiment, GFP- n donated an 11-nucleotide CRL, whereas GFP- m donated a 12-nucleotide CRL to RSV. The same result was obtained: GFP- m was much less frequently targeted than was GFP- n (see File S2 for the raw data).

A bean phytohemagglutinin (PHA) mRNA containing a PTC at codon 79 (PHA-m), which has been used as a nsRNA by researchers dissecting NMD of plants, was used to confirm the low frequency of RSV for longer CRLs. A reciprocal experiment was done to investigate whether RSV targets PHA- m more frequently in comparison to PHA- n. A direct competition assay like that carried out for GFP- m and GFP- n is infeasible. As an alternative, pHB-PHA- m and pHB-PHA- n were each agro-infiltrated into Nicotiana benthamiana using the binary vector pHB. PHA-n, the normal PHA mRNA, was expressed with the same vector. Because the 5′-terminal sequences of PHA- m and PHA- n are identical (Figure 1d), a direct competition assay like that carried out for GFP- m and GFP- n is infeasible. As an alternative, pHB-PHA- m and pHB-PHA- n were each agro-infiltrated independently into a different leaf of the same plant. A. tumefaciens cell cultures carrying pCHF3, which expresses a GFP mRNA, were included at a ratio of 1:1 in each agro-infiltration (Figure 1d). In this way, we used two independent competition assays, one between PHA- m and GFP and the other between PHA- n and GFP, to investigate whether RSV targets PHA- m more frequently in comparison to PHA- n. Sample collection and RSV NP deep sequencing were performed as done above. Similarly, RSV NP mRNA sequences with CRLs derived from PHA- m, PHA- n, and GFP were called PHA-m-NP, PHA-n-NP, and GFP-NP, respectively.

The accumulation of PHA-m-NP relative to GFP-NP (PHA-m-NP/GFP- NP) was compared to that of PHA-n-NP relative to GFP-NP (PHA-n-NP/GFP-NP) (File S2). As shown in Figure 1e, the mean PHA-m-NP/GFP-NP value was about 0.18:1. In contrast, PHA-n-NP/GFP-NP had a mean value of 1.49:1. Thus, like GFP- m, PHA- m was
targeted much less frequently than normal mRNAs by the cap-snatching of RSV.

To investigate how deficiencies in early steps of NMD influence the cap-snatching of RSV from nRNAs, the competition assays described above were carried out in *N. benthamiana* whose UPF1 or SMG7, each encoding a protein component involved in early steps of NMD, had been silenced using virus-induced gene silencing (VIGS).

To do this, a cDNA fragment of UPF1 or SMG7 was cloned into the tobacco rattle virus (TRV)-based VIGS vector pTRV2 (Liu et al., 2002). *A. tumefaciens* cell cultures containing pTRV2-UPF1, pTRV2-SMG7, or pTRV2 carrying a fragment of luciferase (pTRV2-LUC), which was used as a control, were each mixed with cultures containing pTRV1 before being infiltrated to leaves of *N. benthamiana*. At 10 dpi, when UPF1 and SMG7 had been silenced by about 68% and 76% (data not shown), respectively, the *N. benthamiana* was rub-inoculated with RSV. Twenty days after the rub-inoculation, agro-infiltration, sample collection, and deep sequencing of RSV NP mRNA were performed as done above. Because reducing UPF1 or SMG7 expression may enhance the activity of RDR6-mediated gene silencing, which may influence data interpretation, all these assays were done with an *rdr6* RNAi line of *N. benthamiana* (Liu & Chen, 2016; Moreno et al., 2013; Qu et al., 2005).

Before deep sequencing of RSV NP mRNAs, we investigated how the relative accumulation of GFP-m or PHA-m was influenced in UPF1- or SMG7-silenced plants. The accumulation of PHA-m relative to that of PHA-n was detected with reverse transcription-quantitative PCR (RT-qPCR). As shown in Figure 2a, the relative accumulation of PHA-m was increased 2.2-fold in UPF1-silenced plants in comparison to control plants (plants preinfected by TRV-LUC). In contrast, its relative accumulation was unchanged in SMG7-silenced plants. The relative accumulation of GFP-m and GFP-n was studied with a different approach. In this approach, total RNA...
extracted from leaf patches co-expressing GFP-m and GFP-n was reverse transcribed using a random primer. The cDNA was PCR amplified using a primer pair (corresponding to a region underlined in Figure 1a) flanking codon 3 of GFP-m/GFP-n and the PCR amplicon was deep sequenced. Because GFP-m and GFP-n differ by one nucleotide at codon 3, the RT-PCR sequences corresponding to the two mRNA molecules can be distinguished from each other. As shown in Figure 2b, this experiment showed that the accumulation of GFP-m was increased 2.63-fold in SMG7-silenced plants but was unchanged in UPF1-silenced plants.

The observation that silencing of UPF1 or SMG7 each influenced the relative accumulation of only one nsRNA is unexpected. However, this is consistent with a recent report showing that UPF1 or SMG7 may each regulate an overlapping but different set of cellular transcripts (Raxwal et al., 2020). Alternatively, the residual UPF1/SMG7 after VIGS may be still enough to commit GFP-m/PHA-m to NMD. Whatever the possibility, this offered us a unique opportunity to see whether the effects of UPF1/SMG7 on the frequency by which RSV targets GFP-m/PHA-m correlate with their effects on the accumulation of the two nsRNAs.

The values of GFP-m-NP/GFP-n-NP are presented in Figure 2c (raw data in File S2). The mean GFP-m-NP/GFP-n-NP value in UPF1-silenced plants was comparable to that in control plants. In contrast, the mean GFP-m-NP/GFP-n-NP value in SMG7-silenced plants was about twofold higher than that in control plants. Notably, the number of GFP-m-NP is larger than that of GFP-n-NP in data sets obtained from SMG7-silenced plants. The values of PHA-m-NP/GFP-NP and PHA-n-NP/GFP-NP are presented in Figure 2d (raw data in File S2). As shown, PHA-n-NP/GFP-NP values in all plants are comparable to each other. PHA-m-NP/GFP-NP values in SMG7-silenced plants were comparable to those in control plants, indicating that silencing of SMG7 did not affect the frequency by which RSV targets PHA-m. However, the mean PHA-m-NP/GFP-NP value was increased about fourfold in UPF1-silenced plants relative to those in control plants.

These data indicated that the effects of UPF1/SMG7 on the frequency by which RSV targets GFP-m/PHA-m correlate well with their effects on the accumulation of the two nsRNAs. To put it in other words, the frequency of RSV to target a nsRNA is increased in plants that had lost the ability to commit that nsRNA to NMD.

Typically, RSV causes mosaic, yellowing, and curling of upper leaves in N. benthamiana. These symptoms seemed to be milder in UPF1- or SMG7-silenced plants than in control plants (Figure 3a). However, UPF1- or SMG7-silenced plants showed a decrease in their width (Figure 3b). This symptom was not observed in control plants, nor in UPF1- or SMG7-silenced plants that had not been infected with RSV (Figure 3b; data not shown).
of UPF1 or SMG7 had influenced the susceptibility of N. benthamiana to RSV, the accumulation of the virion-sense and complementary-sense RSV RNA3 (vRNA3 and vcRNA3) in UPF1- or SMG7-silenced plants was detected using a strand-specific RT-qPCR adapted from Kawakami et al. (2011). Silencing of SMG7 significantly increased the accumulation of vcRNA3. The accumulation of vRNA3 was also discernibly increased, although this was not supported by statistical analysis. VIGS of UPF1, on the other hand, significantly increased the accumulation of both vRNA3 and vcRNA3 (Figure 3c,d). Considering the great variation of RSV accumulation in each plant, each sample contained pooled upper leaves of six to eight different plants in northern blotting. As shown in Figure 3e, the average accumulation level of RSV RNA3 was much higher in UPF1- or SMG7-silenced than in control plants. Altogether, these data indicated that silencing of either UPF1 or SMG7 increased the susceptibility of N. benthamiana to RSV.

In all, by using a recently established system that allows us to artificially present defined mRNAs to RSV, this study for the first time investigated the cap-snatching of a plant bunyavirus from nsRNAs. In contrast to a previous report for a hantavirus (Mir et al., 2008), RSV targets nsRNAs much less frequently than it targets normal mRNAs. The frequency of RSV to target nsRNAs was increased in UPF1- or SMG7-silenced plants, indicating that NMD is responsible for the low frequency of RSV to target nsRNAs.

Assuming that nsRNAs are transported to PBs at later stages of NMD in plants, our findings can be interpreted in two different ways. First, RSV performs cap-snatching mainly in the diffuse cytoplasm. Second, RSV performs cap-snatching in PBs but lacks a mechanism to cope with the high rate of nsRNA degradation in PBs. Given that diverse bunyaviruses including one belonging to the same family as RSV have been suggested to use PBs as important sites for cap-snatching (Hopkins et al., 2013), we hypothesize that the second explanation is likely to be correct. However, our data suggest that PBs are not the sole sites for the cap-snatching of RSV, otherwise it will be
difficult to explain the increased frequency of RSV to target nsRNAs in UPF1- or SMG7-silenced plants. Is it possible that neither of GFP-m and PHA-m goes to PBs at later stages of NMD? We cannot rule out this possibility. If this is true, the interpretation of our data becomes a little more complex. However, our conclusion that NMD restricts the frequency of RSV to target nsRNAs seems to be inarguable.

Given the complex interactions between viruses and NMD of their host cells, the mechanisms underlying the increased accumulation of RSV in UPF1- or SMG7-silenced N. benthamiana are uncertain at present (Balistreri et al., 2017; Li & Wang, 2019). A plausible explanation, however, is that nsRNAs as well as other transcripts regulated by NMD were accumulated in these plants. This leads to a larger mRNA pool that is available for RSV to perform cap-snatching. If this explanation is true, our finding points to a novel role of NMD in plant–bunyavirus interactions, that is, NMD may limit the infection of bunyaviruses by posing a constraint to their cap-snatching.

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CONFLICT OF INTEREST
The authors declare that they have no conflict of interest.

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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