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Cap-snatching inhibitors of influenza virus are inhibitory to the in vitro transcription of rice stripe virus

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Abstract

Rice stripe virus (RSV) is one of the most important viral pathogens of rice in East Asia. The transcription of RSV is initiated by cap-snatching, a mechanism shared by influenza virus (IFV). This lends the possibility that antiviral compounds targeting the cap-snatching of IFV, many of which have been commercially available, may inhibit RSV transcription. A convenient and inexpensive system allowing researchers to test this idea, however, has been unavailable to date. Here, we show that purified RSV performs transcription in vitro and the transcription was readily detectable by nested reverse transcription-polymerase chain reaction (RT-PCR). With this system, we tested the effects of 2,4-dioxo-4-phenylbutanoic acid (DPBA) and pimodivir, two well-known IFV cap-snatching inhibitors, as well as ribavirin, a broad-spectrum antiviral compound whose targets remain elusive. In reaction mixtures containing 2.5 ng/μL of purified RSV, DPBA and pimodivir abolished RSV transcription at a concentration of 10 and 100 μM, respectively. In contrast, no inhibitory effect was detected from ribavirin, even at a concentration as high as 400 μM. These results suggest that at least some cap-snatching inhibitors of IFV are inhibitory to RSV transcription. These compounds, which can be identified with the experimental system described here, may serve as starting points in developing antivirals against RSV or related plant viruses.

Keywords: Rice stripe virus, Cap-snatching, Transcription, Antivirals

Background

Rice stripe virus (RSV) is the type species of the genus *Tenuivirus*, which has recently been assigned to the family *Phenuiviridae* of the order *Bunyavirales* (Kormelink et al. 2021). RSV is transmitted in a circulative and propagative manner by the small brown planthopper *Laodelphax striatellus* Fallén. It infects the staple food crop rice, causing severe yield losses in some East Asian countries including China, Japan, and South Korea (Ramirez and Haenni 1994; Falk and Tsai 1998; Xu et al. 2021). As with most other plant viruses, antivirals are unavailable for RSV. The strategies used in RSV management significantly increase the cost of rice production (Xu et al. 2021).

The genome of RSV consists of four single-stranded RNA segments named RNA1, RNA2, RNA3, and RNA4, respectively, in decreasing order of their molecular mass (Xu et al. 2021). RNA1–4 are each bound by multiple copies of the viral nucleocapsid protein (NP), forming distinct ribonucleoprotein (RNP) complexes. Besides NP, each RNP is associated with one copy of the viral RNA-dependent RNA polymerase (RdRp) (Toriyama 1982, 1986; Ishikawa et al. 1989; Sun et al. 2018; te Velthuis et al. 2021). The viral RdRp performs two functions, namely replication and transcription of the genome. Replication generates complementary-sense RNAs (cRNAs).

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The cRNAs, which are assembled to RNPs like viral-sense RNAs (vRNAs), then serve as intermediates to produce progeny vRNAs. Transcription generates mRNAs, which direct the production of viral proteins. For RNA1, only the vRNA is transcribed, producing a mRNA that encodes the viral RdRp (Toriyama et al. 1994). For RNA2–4, both vRNA and cRNA are transcribed, with each resultant mRNA encoding a distinct protein of RSV (Kakutani et al. 1990; Hamamatsu et al. 1993; Takahashi 1993; Zhu et al. 1991). The functions of the proteins encoded by RSV have been studied extensively, as reviewed recently by Xu et al. (2021).

Whereas replication of RSV genomic segments occurs de novo, transcription of them requires primers. The primers are capped RNA leaders (CRLs) cleaved from host cellular mRNAs in a process called cap-snatching (Liu et al. 2018; Olschewski et al. 2020). The cap-snatching is believed to be consisted of three steps. First, a host cellular mRNA is captured by a putative cap-binding domain of the viral RdRp. Second, the host cellular mRNA is cleaved by an endonuclease activity associated with the RdRp (Zhao et al. 2019). Finally, the 5′ product of the endonucleolytic cleavage, which are usually 10–20 nucleotides (nt) in length, is recruited to prime RNP transcription. As a result of cap-snatching, all RSV mRNAs have a non-viral CRL sequence at their 5′ ends (Liu et al. 2018; Olschewski et al. 2020).

The cap-snatching mechanism is shared by almost all segmented negative-sense RNA viruses including the orthomyxovirus influenza virus (IFV) (Decroly et al. 2012). The cap-snatching of IFV has been studied in more detail (Krischuns et al. 2021). Many chemical compounds with inhibitory effects on IFV cap-snatching are now available, with one of them has already been licensed for influenza therapies (Hayden and Shindo 2019; Takashita 2021). Recently, several of these compounds have been shown to be effective against some animal bunyaviruses (Fernández-García et al. 2020; Wang et al. 2020). It is reasonable to assume that this also applies to RSV. Even if these compounds cannot be used immediately in RSV management, they can be used as starting points for the designment of antivirals for RSV. A convenient and inexpensive experimental system that allows researchers to test this idea, however, is unavailable.

In this paper, we describe an experimental system in which purified RSV RNPs perform transcription in vitro and the product of the transcription, which has a CRL acquired from artificially provided capped RNAs, is readily detectable by nested reverse transcription-polymerase chain reaction (RT-PCR). Consequently, the effects of known IFV cap-snatching inhibitors on the transcription of RSV can be tested. With this system, we find that two IFV cap-snatching inhibitors are indeed inhibitory to the transcription of RSV.

Results

Purification of RSV RNPs

RSV RNPs purified from 50 g of RSV-infected rice with the procedure described in the Methods section were resuspended in 0.5 mL of Tris–HCl (pH 8.0, 20 mM). The protein components of the preparation were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). As shown in Fig. 1a, a major band with a size corresponding to RSV NP (about 35 kDa) was observed on the gel after Coomassie brilliant blue staining (Fig. 1a). In Western blotting, this band reacted strongly with an antiserum against RSV NP (Fig. 1b). As a comparison, our previously obtained crude RSV preparations, which had been shown to possess in vitro transcription activity (Lin et al. 2020), were subjected to the same analysis. Multiple bands varying in size were observed, only one of which reacted with the antiserum against RSV NP (Fig. 1a, b). Transmission electron microscopy was conducted to further confirm that RSV RNPs had been obtained. As shown in Fig. 1c, the typical filamentous RNPs of RSV (Ishikawa et al. 1989; Lu et al. 2020) were readily detectable from the preparation obtained here. A bicinchoninic acid (BCA) assay was conducted to quantify protein contents. The results showed that the purified RSV RNP preparation obtained in this study has a protein concentration of about 1.5 μg/μL (data not shown).

Like a recent report from Lu et al. (2020), a band corresponding to RdRp is not detected in SDS-PAGE. Most probably, the content of RdRp obtained with this method is too low relative to that of NP in RSV RNPs.

In vitro transcription of purified RSV RNPs

To investigate whether the purified RSV RNPs could perform transcription in vitro, reaction mixtures were prepared as described in the Methods section. In these reaction mixtures, the genomic RNAs of cucumber mosaic virus (CMV) were artificially provided as CRL donors. According to our current understanding, in vitro transcription of RSV RNPs would generate RSV mRNAs with CMV-derived CRLs at their 5′ termini (Fig. 2a) (Liu et al. 2018; Olschewski et al. 2020). These mRNAs can be detected via nested RT-PCR with primers P1–P3 (Fig. 2a). Figure 2b shows the results for the detection of RSV *NP* mRNAs. A band of expected size was obtained. The intensity of this band is roughly proportional to the amount of RSV in the reaction mixture (Fig. 2b, lanes 1–7). As controls, this band was not detected in reactions in which RSV had been heat inactivated at 90 °C for 5 min, nor in those in which CMV

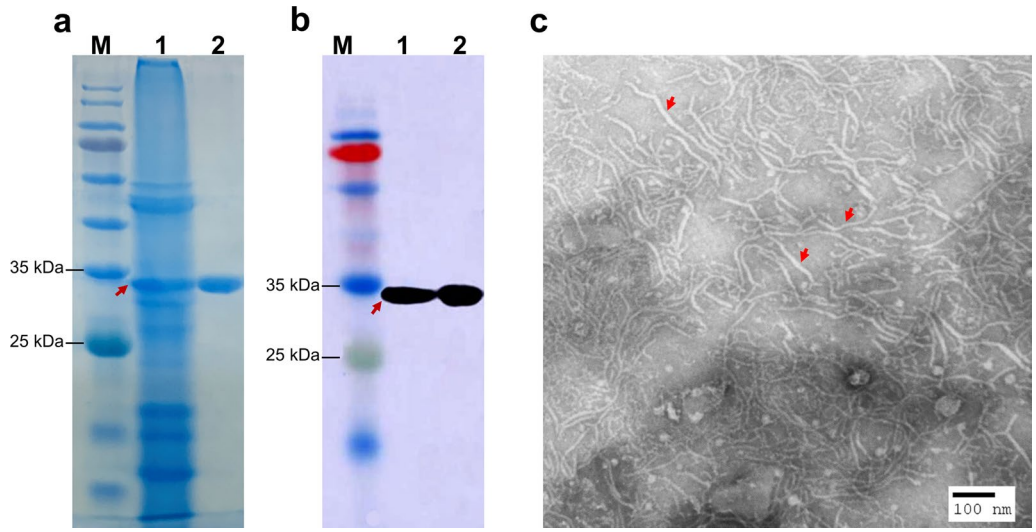

Fig. 1 Purification of RSV. **a** SDS-PAGE analysis of the purified RSV RNPs. Lane 1, crude RSV preparations obtained previously (Lin et al. 2020) were used as a control; Lane 2, RSV RNPs purified in this study. 10 μL of RSV preparations were loaded into each well. The gel was stained with Coomassie brilliant blue after electrophoresis. The arrows indicate the band with a size corresponding to RSV NP. M, molecular maker. **b** Western blotting analysis of the NP of RSV. After separation by SDS-PAGE, the proteins of the RSV preparations were transferred onto a nitrocellulose membrane and the blot was probed with an antiserum against RSV NP followed by a goat anti-rabbit IgG conjugated with HRP. **c** Observation of RSV via transmission electron microscopy. Red arrows indicate representative RSV RNPs. Scale bar, 100 nm

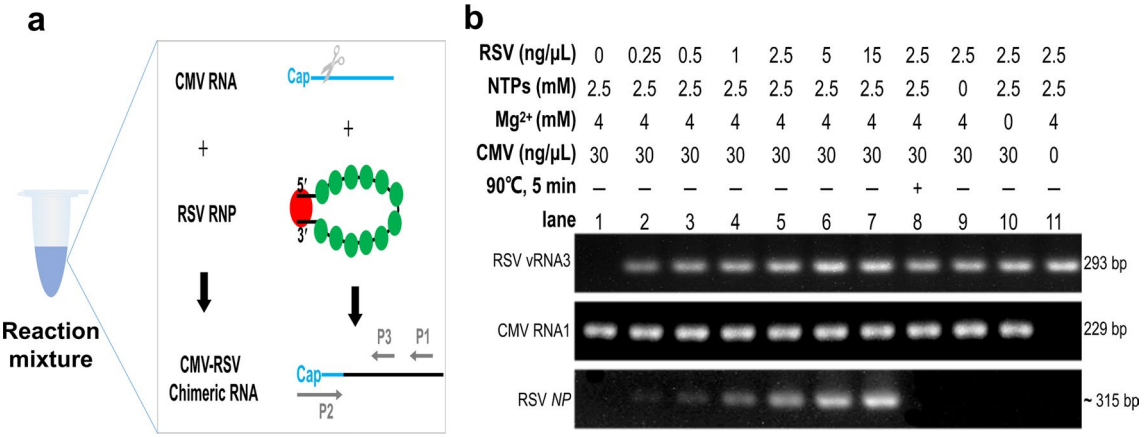

Fig. 2 RSV performs transcription in vitro. **a** A diagram illustrating the strategy to detect in vitro transcripts (the *NP* mRNA). CMV RNAs (the blue line, only one of the CMV RNA segments was shown here) and RSV RNPs are present in the reaction mixture. The RdRp (red ball) of RSV RNPs (only one RNP is shown here) cleaves CMV RNAs, obtaining a CRL (not shown). The CRL is used as a primer to transcribe viral template RNAs, leading to the generation of chimeric RSV mRNAs with a CMV-derived CRL at their 5′ ends, which can be detected by nested RT-PCR using primers 1, 2 and 3 (P1–P3). Primer 2 is a composite primer with ten nucleotides at its 3′-end identical to the 5′ terminus of CMV RNA1. Similar nested RT-PCR was conducted to detect RSV and CMV genomic RNAs. In doing this, all the three primers anneal to the coding region of RSV *NP* (not shown) or CMV RNA1. **b** Gel electrophoresis of the RT-PCR product. The RT-PCR product from each reaction mixture as indicated on top of the gel was electrophoresed in a 1.5% agarose gel before being visualized

RNAs, NTP or $MgCl_2$ were omitted (Fig. 2b), although in all these reactions the vRNA of RNA3 (the template for *NP*) were readily detectable (Fig. 2b).

To further confirm the transcription of the purified RSV RNPs, the RT-PCR product from the reaction with 2.5 ng/μL of RSV (Fig. 2b, lane 5) was recovered from the gel and sequenced after cloning. These sequences

are indeed chimeric RSV mRNAs with CRLs derived from CMV RNAs (Table 1). They showed no differences from those obtained in a previous study in which RSV transcription was performed in vivo with CMV as CRL donors (Yao et al. 2012).

The effect of DPBA on the transcription of RSV

The fact that purified RSV RNPs perform transcription and the transcription is readily detectable with RT-PCR provides us with a convenient and inexpensive experimental system to test the effects of IFV cap-snatching inhibitors on RSV transcription. As the first step, DPBA, one of the several diketo acid derivatives that inhibit IFV cap-snatching, was tested (Tomassini et al. 1994). To do this, different concentrations of DPBA were included in transcription reactions with 2.5 ng/μL of RSV. RSV *NP* mRNA was detected as described above. As shown in Fig. 3, DPBA was obviously inhibitory to RSV transcription. At a concentration of 10 μM, it almost abolished the transcription. The inhibitory effect was alleviated by increasing the amount of RSV in the reaction (Fig. 3, lanes 7 and 8), suggesting that DPBA targets RSV in a dose-dependent manner. The vRNA of RNA3 and the RNA1 of CMV was readily detectable in all reactions, irrespective of the concentration of DPBA, indicating that DPBA did not destabilize the genomic RNAs of RSV or CMV (Fig. 3).

The effect of pimodivir on the transcription of RSV

The inhibitory effect of pimodivir, a cap-snatching inhibitor whose potential use in anti-influenza therapy is under phase 2 study (Finberg et al. 2019), was tested in a similar way. As shown in Fig. 4, pimodivir showed inhibitory effect on the transcription of RSV. However, the effect was not evident until its concentration

Table 1 The sequences of RSV *NP* mRNAs produced in vitro

CMV RNA1	GTTTATTTACAAGAGCGTACGGTTCAATCCCTGC---	Clone number
RSV *NP*	GTTTATTTAC----------ACACAAAGTC---	3
	GTTTATTTACAA----------CACAAAGTC---	1
	GTTTATTTAC-----*ACAC*----------ACACAAAGTC---	2
	GTTTATTTAC-------*ACACAC*------ACACAAAGTC---	1
	GTTTATTTACAAGAGC----------ACACAAAGTC---	1
	GTTTATTTAC-----*ACACAC*--------ACACAAAGTC---	1
	GTTTATTTACAAGAGC----------ACAAAGTC---	2

The first line shows the 5′-terminal sequence of CMV RNA1 (in red). The remaining lines show RSV *NP* mRNAs (and their clone numbers) with their 5′-CRLs derived from CMV RNA1. For RSV *NP* mRNAs, nucleotides matching CMV RNA1 were in red, matching both CMV RNA1 and RSV RNA3 were underlined, matching neither CMV RNA1 nor RSV RNA3, which were supposed to be produced by a mechanism named prime-and-realign (Liu et al. 2018), were italicized. Dotted lines were introduced to align the sequences or to represent omitted nucleotides

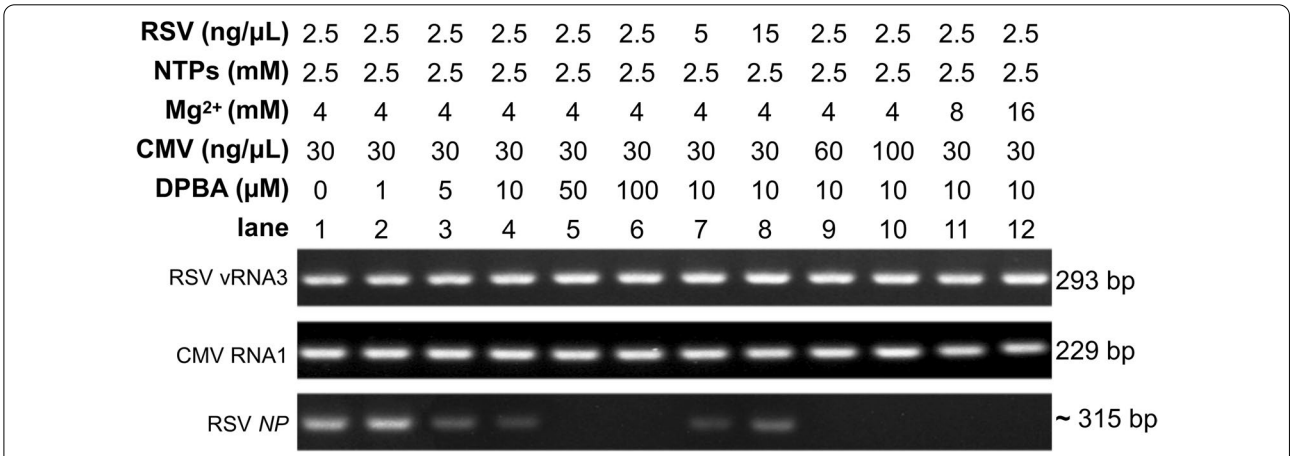

Fig. 3 The effect of DPBA on the transcription of RSV. The chimeric RSV mRNA (RSV *NP*) produced in vitro from each reaction with a mixture as indicated on top of the gel was amplified by RT-PCR. Similar nested RT-PCR was conducted to detect RSV and CMV genomic RNAs. The RT-PCR product was electrophoresed in a 1.5% agarose gel before being visualized

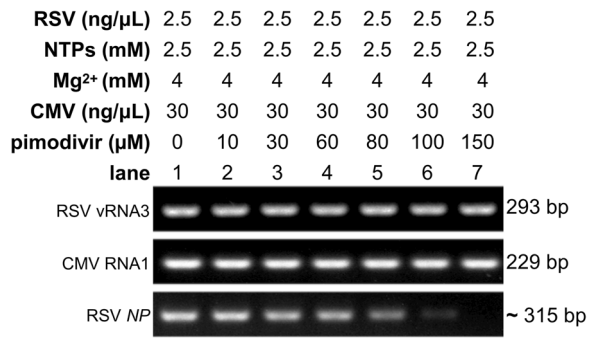

Fig. 4 The effect of pimodivir on the transcription of RSV. The chimeric RSV mRNA (RSV *NP*) produced in vitro from each reaction with a mixture as indicated on top of the gel was amplified by nested RT-PCR. Similar nested RT-PCR was conducted to detect RSV and CMV genomic RNAs. The RT-PCR product was electrophoresed in a 1.5% agarose gel before being visualized

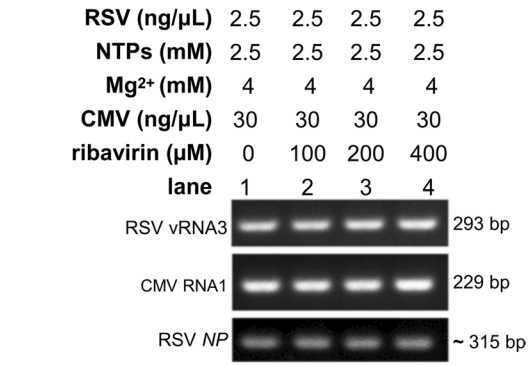

Fig. 5 The effect of ribavirin on the transcription of RSV. The chimeric RSV mRNA (RSV *NP*) produced in vitro from each reaction with a mixture as indicated on top of the gel was amplified by RT-PCR. Similar nested RT-PCR was conducted to detect RSV and CMV genomic RNAs. The RT-PCR product was electrophoresed in a 1.5% agarose gel before being visualized

reached 80 μM in the reaction. Thus, the inhibitory effect of pimodivir seems to be weaker than that of DPBA.

The effect of ribavirin on the transcription of RSV

Ribavirin has been used in therapies against a wide range of human viruses (Graci and Cameron 2006). A recent study demonstrated that ribavirin inhibits RSV acquisition by its insect vector *L. striatellus* (Hajano et al. 2020). This promoted us to test whether ribavirin can inhibit the transcription of RSV. Unexpectedly, based on the results of this study, no inhibitory effect was observed from ribavirin, even at a concentration as high as 400 μM (Fig. 5).

Discussion

Here, we showed that purified RSV RNPs performs transcription in vitro and the transcription can be detected readily by RT-PCR. With this system, we found that two cap-snatching inhibitors of IFV were inhibitory to RSV transcription. However, ribavirin, a compound with a broad-spectrum antiviral activity, could not inhibit RSV transcription, even at very high concentrations.

The finding that purified RSV RNPs are capable of performing transcription is not unexpected. Firstly, in vitro transcription activity has been reported in many negative-sense RNA viruses including those infecting plants (Nguyen et al. 1997; van Knippenberg et al. 2002). It is generally accepted that the RNPs of these viruses possess all activities required for transcription (Sun et al. 2018; te Velthuis et al. 2021). Secondly, an in vitro RNA polymerase activity for RSV has been reported previously, although these earlier studies did not distinguish transcription from replication (Toriyama 1986; Barbier et al. 1992). This study is the first to demonstrate the utility of in vitro transcription as an experimental system to identify compounds that inhibit RSV transcription. Although this experimental system is not quantitative in its present form, it has obvious advantages: (i) it does not require radioactivity as a readout and can be carried out in any labs equipped with basic instruments for molecular cloning; (ii) a very small amount of RSV is used in each reaction, which means that the virus is not a limiting source for the experiment.

The cap-snatching of IFV is a multistep process with each step catalyzed by a distinct domain of the viral RdRp (Reich et al. 2014; De Vlugt et al. 2018). The two cap-snatching inhibitors investigated in this study target different RdRp domains. DPBA targets the endonuclease domain, which is responsible for cleavage of the CRL donor (Dias et al. 2009). Pimodivir targets the cap-binding domain, whose function in cap-snatching is to capture the CRL donor by binding to its cap structure and presenting the CRL to the endonuclease (Guilligay et al. 2008). Our results showed that DPBA is much more inhibitory to RSV than pimodivir. In fact, the effects of DPBA on RSV seem to be comparable to its effects on IFV (Tomassini et al. 1994). This is consistent with the fact that RSV RdRp, like those of all other bunyaviruses characterized to date, has an endonuclease domain very similar to the endonuclease of IFV (Reguera et al. 2010; Holm et al. 2018; Zhao et al. 2019), and the fact that some compounds targeting the endonuclease of IFV have been shown to be inhibitory to the endonuclease activity of some animal bunyaviruses (Fernández-García et al. 2020; Wang et al. 2020). In contrast, it is generally accepted that bunyaviruses including RSV may use cap-binding domain distantly related to that of IFV (Olschewski et al. 2020).

Therefore, it is advisable that further studies aiming to search RSV transcription inhibitors should focus on IFV cap-snatching inhibitors targeting the endonuclease.

At present, it is difficult to test the i*n vivo* effects of the IFV cap-snatching inhibitors on RSV mainly because these compounds are too expensive to be directedly applied to RSV-infected plants. Moreover, it may be impossible to repurpose human drugs to RSV management. Given these limitations, the practical implications of the present work are still obvious for several reasons. Firstly, the IFV inhibitors effective against RSV can be used as starting points in designing antivirals against RSV. Secondly, many IFV cap-snatching inhibitors have been reported, but only a few of them can be used in humans (Hayden and Shindo 2019; Takashita 2021). The compounds that cannot be used in humans may be used in plants. Thirdly, the experimental system described here can be used to identify new antiviral compounds besides testing the effects of known ones. In the second sense, it is noteworthy that the inhibitory effects of DPBA on IFV cap-snatching was discovered more than 20 years ago (Tomassini et al. 1994). It seems that this compound has been abandoned by researchers searching for anti-influenza therapies.

Besides RSV, many other devastating plant viruses, such as those belonging to the genus *Tospovirus*, use the cap-snatching mechanism to initiate transcription of their mRNAs (Kormelink et al. 2021). It is not unconservative to assume that IFV cap-snatching inhibitors are also effective against these plant viruses. In this sense, the experimental system described here may have more broad applications.

Conclusions

At least some cap-snatching inhibitors of IFV inhibit the transcription of RSV. The experimental system described in this study can be used to identify these compounds.

Methods

RSV purification

A Jiangsu isolate of RSV was maintained and propagated in rice plants. The inoculated rice plants were grown in the field and those showing typical RSV symptoms were harvested for virus purification. Briefly, crude RSV preparations were obtained with a procedure described by Wang et al. (2014). The crude preparations were centrifugated through a 10–40% sucrose density gradient as described previously (Toriyama 1986; Barbier et al. 1992). The bands containing RSV, as detected by SDS-PAGE, were collected before being resuspended in Tris–HCl buffer. The protein content of the solution was quantified using a BCA Protein Quantification Kit (Vazyme, China) according to manufacturer's instructions. RSV was stained with uranyl acetate solution before being examined and imaged with a transmission electron microscope (H-7650; Hitachi, Tokyo, Japan).

In vitro transcription

In vitro transcription of purified RSV was set up according to previous reports with modifications (Nguyen et al. 1997; van Knippenberg et al. 2002; Lin et al. 2020). Briefly, RSV was incubated for 90 min at 30 °C in a final reaction mixture containing 10 mM Tris–HCl (pH 8.0), 2 mM DTT, 2.5 mM of each NTP, 2 U of RNase inhibitor (Vazyme, China), 4 mM $MgCl_2$, and 30 ng of CMV RNA (the artificial cap donor extracted from CMV particles) in the presence or absence of IVF RdRp inhibitors. In some reactions, the concentrations of CMV RNA, NTP or $MgCl_2$ were varied as indicated in the text. DPBA(CAS:5817-92-5) was purchased from Shanghai Yuanye Bio-Technology, China. Pimodivir (CAS:1629869-44-8) was purchased from ProbeChem, and ribavirin (CAS:36791-04-5) was purchased from Solarbio. All the compounds were dissolved in dimethyl sulfoxide (DMSO) to different final concentrations so that the amount of DMSO is equal in reactions with different concentrations of these compounds.

Detection of in vitro transcription products via nested RT-PCR

The in vitro transcription of RSV generates chimeric RSV mRNAs with a CRL, which is derived from CMV RNA (Fig. 2a). These transcripts can be detected via nested RT-PCR as described previously (Yao et al. 2012; Liu et al. 2016; Lin et al. 2020). In brief, the reaction mixture was purified with VAHTS RNA Clean Beads (Vazyme, China), and the purified RNA was dissolved in 20 μL of RNAse-free water. An aliquot of 2 μL of the purified RNA was reverse transcribed with primer 1 (5′-TACCTCGAC ACCAAGGTCGAAGCCTCAG-3′), which specifically anneals to a sequence within the coding region of RSV *NP*, yielding 20 μL cDNA. Two μL of the cDNA was PCR amplified with primer 2 (5′-GTACCCGGATCCGTTTAT TTAC-3′) and primer 3 (5′-TCCTTGGCCAGTGTGTCA CCACCTTTGT-3′) as the forward and reverse primers, respectively. Primer 2 is a composite primer with ten nucleotides at its 3′-end identical to the 5′-terminus of CMV RNA1. Primer 3 lies upstream of primer 1 (Fig. 2a). Thermocycler settings for the PCR, with a final reaction volume of 20 μL, were as follows: 3 min at 94 °C, followed by 32 cycles of 30 S at 94 °C, 30 S at 55 °C, 30 S at 72 °C, and a final extension for 10 min at 72 °C.

As controls, RSV and genomic RNAs were detected with similar nested RT-PCR experiments. In detecting RSV genomic RNAs, primer 4 (5′-CATCTAAGCCGC AACCATTCCTCCAG-3′), which anneals to a sequence

within the templating region of RSV NP (RSV RNA3), was used in reverse transcription, and primer 5 (5′-GGT CGAAGCCTCTGTGCTTCCAGC-3′) and primer 6 (5′-CCAACAAGCCAGCCACTCTAGCTG-3′), both of which were upstream of primer 4, were used as forward and reverse primers, respectively, in PCR. In detecting CMV genomic RNAs, primer 7 (5′-AACGTTCCGGAT GTAGACCTTACG-3′), which anneals to a sequence within the templating region of CMV RNA1, was used in reverse transcription, and primer 2 and primer 8 (5′-TTGATGCTGTAATTGCTCCTCGAG-3′) were used as forward and reverse primers, respectively, in PCR. Nested RT-PCR products from each reaction were electrophoresed in a 1.5% agarose gel after being mixed with dilutions of SYBR Gold stain.

Abbreviations

BCA: Bicinchoninic acid; CMV: Cucumber mosaic virus; cRNA: Complementary-sense RNA; CRL: Capped RNA leader; DPBA: 2,4-dioxo-4-Phenylbutanoic acid; IFV: Influenza virus; kDa: Kilodaltons; Mg^{2+}: Magnesium ion; $MgCl_2$: Magnesium chloride; mRNA: Messenger RNA; NP: Nucleocapsid protein; nt: Nucleotides; NTP: Nucleotriphosphate; RdRp: RNA-dependent RNA polymerase; RNA: Ribonucleic acid; RNP: Ribonucleoprotein; RSV: Rice stripe virus; RT-PCR: Reverse transcription-polymerase chain reaction; SDS-PAGE: Sodium dodecyl sulfate–polyacrylamide gel electrophoresis; vRNA: Viral-sense RNA.

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Author contributions

ZW, ZD, GW, and FY designed the research. WL, QZ, and WZ performed the research. ZD and WL analyzed the data and wrote the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

Not applicable.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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