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Inherent properties not conserved in other tenuiviruses increase priming and realignment cycles during transcription of Rice stripe virus

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ABSTRACT

Two tenuiviruses *Rice stripe virus* (RSV) and *Rice grassy stunt virus* (RGSV) were found to co-infect rice with the same reovirus *Rice ragged stunt virus* (RRSV). During the co-infection, both tenuiviruses recruited 10–21 nucleotides sized capped-RNA leaders from the RRSV. A total of 245 and 102 RRSV-RGSV and RRSV-RSV chimeric mRNA clones, respectively, were sequenced. An analysis of the sequences suggested a scenario consistent with previously reported data on related viruses, in which capped leader RNAs having a 3′ end complementary to the viral template are preferred and upon base pairing the leaders prime processive transcription directly or after one to several cycles of priming and realignment (repetitive prime-and-realign). Interestingly, RSV appeared to have a higher tendency to use repetitive prime-and-realign than RGSV even with the same leader derived from the same RRSV RNA. Combining with relevant data reported previously, this points towards an intrinsic feature of RSV.

1. Introduction

Messenger RNAs (mRNAs) in a eukaryotic cell normally contain a 7-methylguanosine (^{m7}G) cap at their 5′ termini (Shatkin, 1976). The cap plays important roles in stability and translation of the mRNAs (Darnell, 1979; Filipowicz et al., 1976; Schibler and Perry, 1977). Viruses depend on the host translational machinery to produce their proteins, but most of them cannot use the cellular RNA capping machinery (Furuichi and Shatkin, 2000; Decroly et al., 2012). Many viruses are able to cap their own transcripts, for which the viral RNA polymerase contains a methyl-transferase (MT) activity, or they rely on alternative (cap-independent) translation initiation strategies. Segmented, negative-sense RNA viruses lack a MT activity but provide their messenger RNAs with a cap-structure by employing a highly conserved mechanism called cap snatching (Decroly et al., 2012). During this process, the viral RNA polymerase cleaves short, capped RNA leader sequences from host cellular mRNAs and uses them to prime genome transcription. As a result all viral mRNAs are chimeric in nature, and contain a non-viral, heterogeneously sized leader sequence at their 5′ termini (Decroly et al., 2012). The mechanism of cap-snatching has been most intensively studied for influenza virus (Bouloy et al., 1978; Dhar et al., 1980; Dias et al., 2009; Reich et al., 2014; Sikora et al., 2014; Koppstein et al., 2015; Gu et al., 2015) and being indispensable for the virus, has been an important target for development of antivirals (Das et al., 2010; Decroly et al., 2012). Although cap-snatching has meanwhile been established for most (plant- and animal infecting) segmented, negative-strand RNA viruses (Decroly et al., 2012) and studied in some more detail for several viruses (Duijsings et al., 2001; Mir et al., 2008), still many of the mechanistic details remain unknown.

During cap-snatching, viral mRNA leader sequences have also revealed the presence of repetitive sequences between the 5′ non-viral leader sequence and the viral mRNA sequence. The presence of these sequences have been proposed to result from a prime-and-realign event (Vialat and Bouloy, 1992; Jin and Elliott, 1993; Garcin et al., 1995; Geerts-Dimitriadou et al., 2011a, 2011b; Koppstein et al., 2015). This mechanism, though, has first been proposed for *Hantaan bunyavirus* to explain how the viral RNA polymerase could initiate (anti)genome and mRNA synthesis with GTP (Garcin et al. 1995). This mechanism required internal priming and extension for a few nucleotides, followed by a re-alignment of the extended leader sequence several nucleotides backwards.

While the reason for prime-and-realign is still unclear, the requirement for base complementarity between the 3′ end of a

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capped RNA leader and the viral RNA template has remained another matter of debate. While several studies supported the idea of alignment by virtue of base pairing (Dobie et al., 1997; Garcin et al., 1995; Jin and Elliott, 1993) others did not support this idea (Hagen et al., 1995; Krug et al., 1980). In the past decade extensive studies performed on this subject with *Tomato spotted wilt tospovirus* (TSWV) and *Influenza A virus* have shown that both viruses exhibit a preference for capped RNA leader sequences of a certain size and with sequence complementarity to the viral RNA template (Duijsings et al., 2001; Van Knippenberg et al., 2005;). These findings also have strengthened the idea that cleavage of host cellular mRNAs does not (have to) occur immediately after the ultimate 3′ residue from the so called non-viral leader sequence but more likely occurs further downstream, to support priming by basepairing.

Even in times when high throughput sequencing is becoming more common and larger amounts of viral transcript are analyzed, snatched leader RNAs are generally short and heterogeneous in sequence which prevents identification of the host cellular mRNAs from which the capped-RNA leaders have been cleaved (Sikora et al., 2014; Koppstein et al., 2015; Gu et al., 2015). Analysis of these leader sequences in light of their putative cleavage site, base pairing requirement and prime-and-realign during cap-snatching, thus remains difficult.

Tenuiviruses are segmented, negative-sense RNA viruses infecting plants of the *Gramineae* (Falk and Tsai, 1998) and members of this genus, including *Rice stripe virus* (RSV) and *Rice grassy stunt virus* (RGSV), are major viral pathogens of rice in southeast Asia (Ramirez and Haenni, 1994; Hibino, 1996; Falk and Tsai, 1998). Although they are lacking an envelope membrane, tenuiviruses are phylogenetically related to viruses of the family *Bunyaviridae*, and in specific to those from the genus *Phlebovirus*. Notably, tenuiviruses and phleboviruses share the highly conserved 5′ (5′-ACACAAAG) and 3′ (CUUUGUGU-3′) termini in their genomic RNA segments (Falk and Tsai, 1998). While tenuiviruses also use cap-snatching (Huiet et al., 1993; Ramirez et al., 1995; Shimizu et al., 1996), this mechanism still has only been poorly characterized for these viruses.

More recently, studies on cap-snatching during a mixed infection of RSV and *Cucumber mosaic virus* (CMV) in *Nicotiana benthamiana* indicated a correlation between the occurrence of prime-and-realign and the size of CMV leaders snatched (Yao et al., 2012). The study also found a remarkably high frequency of RSV to use repetitive prime-and-realign (one to several rounds of prime-and-realign) when priming transcription using CMV leaders. However, during a similar study with the *Maize stripe virus* (MStV) (Estabrook et al., 1998) in a mixed infection of barley with the *Barley stripe mosaic* hordeivirus (BSMV), the occurrence of repetitive prime-and-realign was hardly observed. Because the two studies were performed using very different systems, the factors underlying this discrepancy are elusive.

In this study, we established a system that allows us to analyze the cap-snatching process of the two tenuiviruses RSV and RGSV in parallel in their natural host rice. To this end, both viruses were co-infected on rice with the reovirus *Rice ragged stunt virus* (RRSV), another important rice-infecting virus (Hibino, 1996). Our studies showed that during the co-infection, both tenuiviruses snatch leader RNAs from RRSV in a similar size range, with the occurrence of priming on the ultimate (U_1) or penultimate (G_2 and/or U_3) viral template residues, followed by an immediate extension or single- or multiple prime-and-realign events. Importantly, the results strongly indicate that RSV uses repetitive prime-and-realign with a frequency significantly higher than RGSV, pointing towards an intrinsic feature of this virus.

2. Results

2.1. Both RSV and RGSV snatch RNA leaders from co-infecting RRSV

Since reovirus transcripts are capped (Shatkin, 1974; Furuichi et al., 1975; Hibino, 1996; Furuichi, 2015) and several plant-infecting reoviruses are prevailing viral pathogens of rice, we tested whether RSV and RGSV would use capped RNA leaders from these viruses during a co-infection. To this end, rice was co-inoculated with the oryzavirus RRSV and RSV or RGSV. The oryzavirus RRSV was chosen because its co-infection of rice with RGSV was earlier reported (Ling et al., 1978, Nguyen et al., 2015). Reverse transcriptase-PCR (RT-PCR) amplification on total RNA preparations from the infected rice plants, using primers specific for each of these viruses, confirmed that some plants were co-infected with the reovirus and tenuivirus (data not shown). In a next step a nested RT-PCR was performed, similarly as reported previously (Estabrook et al., 1998; Duijsings et al., 1999; Yao et al., 2012; Fig. 1), to detect the presence of *NP* and *NCP* mRNAs from RSV and RGSV respectively, containing a 5′ capped RRSV leader sequence. To distinguish between different leader sequences from RRSV mRNAs, 4 specific primers were designed (10 nucleotides in size) that would detect the presence of leaders from RRSV RNA1 and 5 or RNA2, 3 and 10, or RNA 4, 7, 8 and 9 or RNA6 (Fig. 1). As negative controls, total RNA isolated from rice plants singly infected with RRSV was mixed with RNA preparations from rice plants singly infected by RGSV or RSV, respectively. While no products were amplified from the negative controls (Fig. 2A–D, lanes 2, 4, 6 and 8), all RT-PCR reactions on RNA from the mixed infections yielded products of expected sizes (Fig. 2A–D, lanes 1, 3, 5 and 7).

To confirm the identity of the RT-PCR amplified products as tenuiviral transcripts harboring RRSV leader sequences, and to further analyze the process of cap-snatching with RSV and RGSV, fragments of the expected sizes were recovered and after cloning sequenced. In total, 347 independent clones were obtained, 81 for RGSV *NCP* (Table 1A), 164 for RGSV *NP* (Table 1B), 60 for RSV *NCP* (Table 1C) and 42 for RSV *NP* (Table 1D), respectively. A closer look at those sequences revealed that all contained a 5′ leader sequence derived from different RNA segments of RRSV (Table 1). In 95% of all the clones at least one additional nucleotide from RRSV was observed right following the RRSV specific forward primer sequence (Fig. 1B, Table 1). These data clearly demonstrated that during a mixed infection in their natural rice host, RSV and RGSV are able to use RRSV as a source for capped-RNA leaders to prime genome transcription.

2.2. Properties of RRSV-RSV and RRSV-RGSV chimeric mRNAs are consistent with base paring and prime-and-realign during tenuiviral cap-snatching

Although many of the mechanistic details of cap-snatching still remain unknown, several studies have indicated that the ability of capped-RNA leader sequences to base pair with the 3′- ultimate residues of the viral RNA template stimulates their usage as cap donor (Duijsings et al., 2001; van Knippenberg et al., 2005; Geerts-Dimitriadou et al., 2011a, 2011b). Priming in those situations is not restricted to the first viral RNA template residue but may also happen on the second or even third viral residue in cases when the leader has a multi-base complenmentarity with the viral template. The latter would give rise to mRNAs in which the first viral residue, or even two or three viral residues is missing (Duijsings et al., 2001). However, this can be repaired by the prime-and-realign mechanism which sometimes even results in production of mRNAs with extra nucleotides at the junction between the host leader and the viral sequence (Fig. 3). Transcript sequences from both RSV