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Short communication

Characterization of an endonuclease in rice stripe tenuivirus Pc1 in vitro

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ABSTRACT

Rice stripe tenuivirus (RSV) initiates its mRNA transcription by using the cap-snatching mechanism during which an endonuclease activity is required for the cleavage of host mRNA. In this study, we aim to characterize the endonuclease in RSV. Sequence alignment revealed the presence of a cap-snatching endonuclease domain in RSV Pc1. Expression and in vitro enzymatic activity assay demonstrated that this domain indeed had a manganese-dependent endonuclease activity. The enzyme could efficiently degrade ssRNA with preference for unstructured ssRNA, but not DNA. Mutations in the endonuclease domain allowed the identification of four key residues (D547, D567, E585 and K604). The endonuclease of RSV was similar but not identical to other known viral endonucleases, suggesting that RSV endonuclease may have some distinct catalytic characteristics.

Rice stripe tenuivirus (RSV) is the causative agent of rice stripe disease and mainly transmitted by the insect vector small brown planthopper (SBPH) in a persistent-propagative manner, which results in significant yield losses in rice production in many East Asian countries (Cho et al., 2013). RSV is a negative-sense single-stranded RNA virus (NSV) with a four-segmented genome containing seven open reading frames (ORFs) (Falk and Tsai, 1998). RNA1 is negative sense and has one ORF coding for a large protein Pc1 (~336.8 kDa), which contains the conserved motifs of RNA-dependent RNA polymerase (RdRp) and thus is considered to be the viral RdRp responsible for transcription and replication of viral genes (Toriyama et al., 1994). RSV RdRp is considered to be processed posttranslationally into a 230-kDa polypeptide and its RNA polymerase activity requires Mg^{2+}, Mn^{2+} or Fe^{2+} but not Na^{+}, K^{+} or NH_4^{+}(Toriyama, 1986). RNA2, RNA3 and RNA4 are ambisense in coding strategy and each contains two ORFs in mutually opposite orientations (Hamamatsu et al., 1993).

Analysis of the 5′-termini of RSV mRNAs revealed the presence of 10–23 non-viral nucleotides (Shimizu et al., 1996; Liu et al., 2018). These non-viral nucleotides were derived from host cellular mRNA and used as the primer by the virus to drive the transcription of viral mRNAs, suggesting that RSV uses a cap-snatching mechanism to initiate transcription of its mRNAs. Cap-snatching involves binding of host capped mRNAs by a viral RdRp and subsequent cleavage of these RNAs several nucleotides downstream of the 5′-cap by a viral endonuclease activity to generate short capped RNAs, which are used as primers for the synthesis of viral mRNAs. This mechanism was first demonstrated for influenza A virus (IAV) of the family *Orthomyxoviridae* (Plotch et al., 1981) and subsequently characterized in other segmented NSVs of the family *Arenaviridae* (Morin et al., 2010) and the order *Bunyavirales* (Decroly et al., 2012). In influenza virus, the endonuclease domain resides in the PA subunit of hetero-trimeric polymerase and is a member of the PD-(D/E)xK nuclease superfamily (Dias et al., 2009). In other segmented NSVs, similar endonucleases have recently been identified in the N-terminus of monomeric polymerases (L proteins) (Morin et al., 2010; Reguera et al., 2010; Wallat et al., 2014; Devigot et al., 2015; Fernández-García et al., 2016; Reguera et al., 2016; Rothenberger et al., 2016; Rosenthal et al., 2017).

RSV belongs to the genus *Tenuivirus* that is recently classified into the family *Phenuiviridae* under the order *Bunyavirales* (Lefkowitz et al., 2018). Employment of cap-snatching for viral mRNA transcription implies the presence of an active endonuclease in RSV. However, the endonuclease has not been characterized. Here we aim to present experimental evidence for the existence of an endonuclease in RSV and further investigate its catalytic properties using biochemical approach. By comparing with other characterized endonucleases, we intend to find the similarities and possible differences in the catalytic activity of these cap-snatching endonucleases.

Blast analysis of the sequence in NCBI database showed that RSV Pc1 has sequence homology with RdRps of some members in the family *Phenuiviridae*. Amino-acid sequence alignments of RSV Pc1 and its homologous proteins revealed that the 430-680aa region of RSV Pc1 possessed a highly conserved motif (H….PD….E….K) (Fig. 1). This motif was identified as the key active site residues for cap-snatching endonucleases in many segmented NSVs (Dias et al., 2009; Reguera

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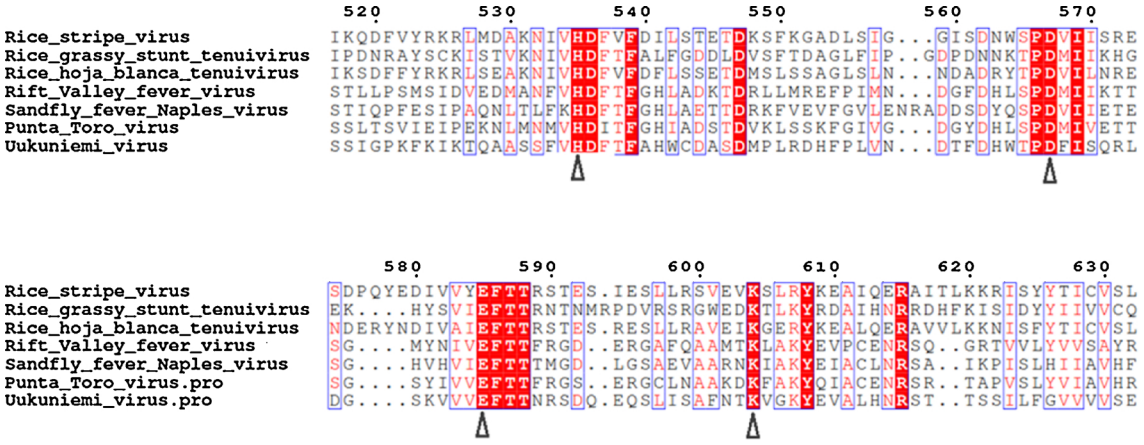

Fig. 1. Amino acid sequence alignment of the N-terminal region of the putative RdRps from viruses in the family *Phenuiviridae*. Sequence alignments were performed with Clustal W and drawn with Espript (http://espript.ibcp.fr/ESPript/cgi-bin/ESPript.cgi.). Residues in a solid red background are identical between seven sequences. The black triangles indicate the key conserved residues for endonuclease activity. Sequences used for alignment were amino acid sequences of RdRps from rice stripe virus (Acc. No. 68333), rice grassy stunt tenuivirus (Acc. No. NP_058528), rice hoja blanca tenuivirus (Acc. No. YP_009449440), rift valley fever virus (Acc. No. YP_003848704), sandfly fever Naples virus (Acc. No. AEL29668) and Uukuniemi virus (Acc. No. NP_941973), respectively (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

et al., 2010, 2016), which belongs to PD….(D/E)XK nuclease superfamily. This result along with previous structure based sequence alignment of bunyaviruses L proteins (Reguera et al., 2010) suggested the presence of an endonuclease domain in RSV Pc1.

To verify whether RSV Pc1 has an endonuclease activity, the gene fragment encoding the putative endonuclease domain (431-680 aa, En) was RT-PCR amplified from total RNA extracted from RSV-infected rice leaves and cloned into pMAL-c2X to generate pMAL-En. The plasmid pMAL-En was then transformed into the *E. coli* strain TB1 (New England Biolabs) cells for En expression with inducing with 0.3 mM IPTG. Two hours later, cells were harvested by centrifugation, resuspended in column buffer (20 mM Tris−HCl pH7.4, 200 mM NaCl and 1 mM EDTA), and disrupted by sonication. The lysate was centrifuged at 10,000 × *g* for 30 min at 4 °C and the MBP-fused proteins in the supernatant were purified using amylose affinity chromatography according to the manufacturer's instructions. The purity of the obtained protein was evaluated by 10% SDS-PAGE gels. One protein band with molecular weight ~68 kDa examined by SDS-PAGE (Fig. 2) indicated that RSV En was successfully expressed in *E. coli* and purified with a high level of purity. The concentration of purified En was then determined by BCA Protein Assay Kit (Tiangen).

The cap-snatching endonuclease activity has been previously shown to depend on divalent cations, usually manganese (Morin et al., 2010; Wallat et al., 2014; Reguera et al., 2016; Fernández-García et al., 2016). Therefore, the endonuclease activity of RSV En was tested in the presence of 1 mM Mn^{2+} using a 35-nt unstructured U-rich ssRNA (5′-GGCCAUCCUGCUCUUUUCCCU$_7$CU$_7$-3′) as substrate. MBP and the residues 1-180 of La Crosse orthobunyavirus (LACV) L protein (LC180) were used as a negative and positive control, respectively. 5 μM of purified proteins were incubated with 50 ng/μL RNA at 37°C in a final volume of 10 μL reaction buffer (20 mM Tris−HCl pH8.0, 100 mM NaCl) with 1 mM Mn^{2+}. After incubation, the reaction products were assessed by electrophoresis on a 15% polyacylamide gel containing 8 M urea and stained with methylene blue. The results showed that RSV En as well as the positive control LC180 could degrade U-rich ssRNA, while the negative control MBP was not able to degrade RNA (Fig. 3A), indicating that RSV En had a nuclease activity. Then, the ion dependence of the nuclease activity was investigated. En was inactive in the absence of divalent ions. Addition of Mn^{2+} stimulated the nuclease activity, while other divalent ions tested (Mg^{2+} and Ca^{2+}) had no effect on En nuclease activity (Fig. 3B), demonstrating that the enzymatic activity was dependent on Mn^{2+} but not Mg^{2+} or Ca^{2+}.

In order to investigate substrate specificity of RSV En, we first

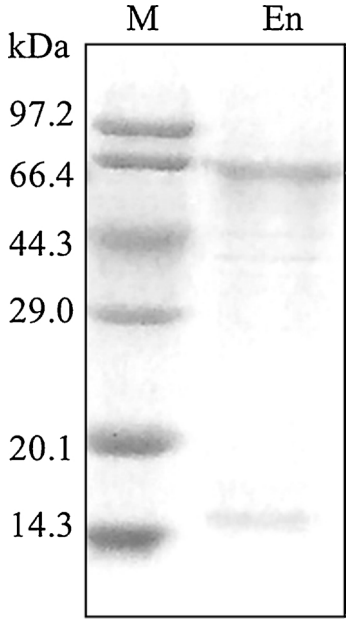

Fig. 2. SDS-PAGE analysis of purified RSV endonuclease domain. The putative endonuclease domain (431–680 aa, En) of RSV was expressed in *E. coli* and purified by affinity chromatography.

compared the enzymatic activity on four different substrates: unstructured U-rich ssRNA, high structured tRNA (Solarbio), 5′-capped mRNA of 271-nt and uncapped mRNA with the same sequence as capped mRNA. Capped and uncapped mRNA were synthesized from previously generated pMD-S4-5′ (Zhao et al., 2015) by in vitro transcription with the mMESSAGE mMACHINE kit (Ambion). After 30 min incubation, unstructured U-rich ssRNA were completely degraded, while highly structured tRNA was partially degraded (Fig. 3), suggesting that the enzyme had a preference for the unstructured RNA. Rapid degration of both capped and uncapped mRNA transcripts (Fig. 3) revealed that the cap structure was not necessary for the cleavage of RNA. Next, we checked the cleavage of En on DNA. A 32-nt ssDNA (5′-CTATTCGATGATGAAGATACCCCACCAAACCC-3′) and a linear 750 bp PCR fragment were used as ssDNA and dsDNA substrates, respectively. MBP and DNaseI were used as a negative and positive control, respectively. In contrast to ssRNA, neither ssDNA nor dsDNA

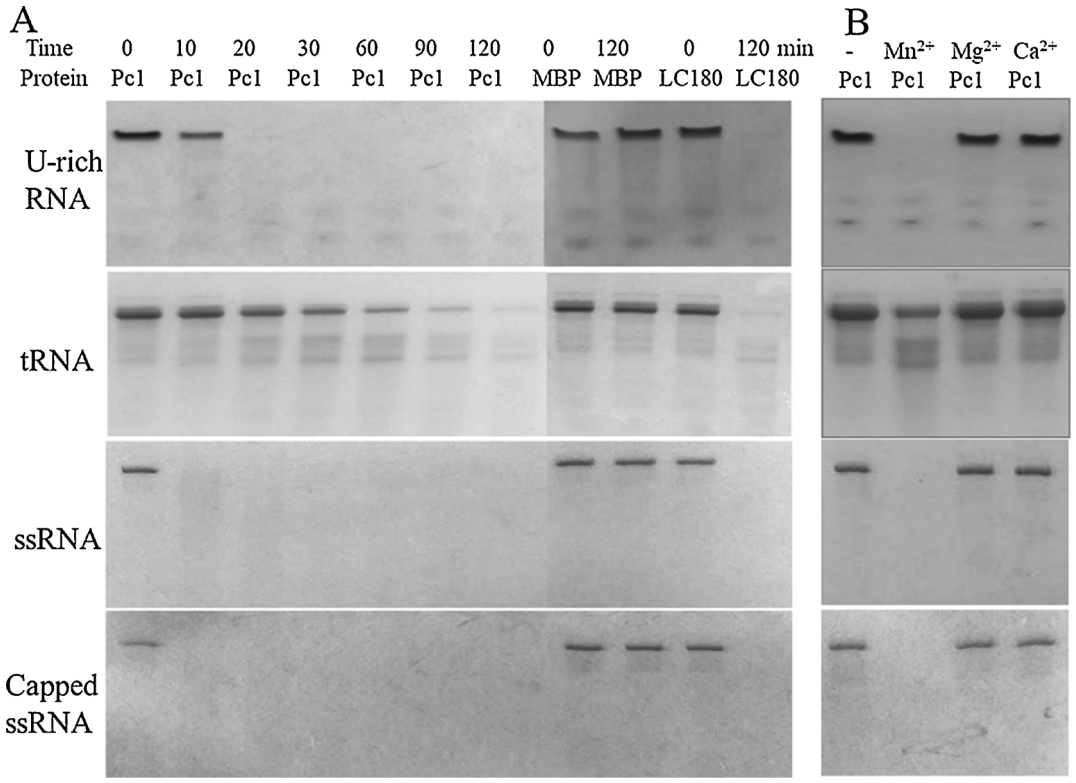

Fig. 3. RSV En Endonuclease activity on RNA. (A) Time series of in vitro degradation assay of RSV En on U-rich ssRNA, tRNA, capped mRNA and uncapped mRNA. Purified RSV En was incubated with substrates at 37℃ in the presence of 1 mM Mn^{2+}. The incubation was stopped by adding loading buffer after 10, 20, 30, 60, 90 and 120 min. MBP and the residues 1–180 of LACV L protein (LC180) were used as a negative and positive control, respectively. The reaction products were assessed by electrophoresis on a 15% denatured polyacylamide gel (U-rich ssRNA and tRNA) or a 10% denatured polyacylamide gel (capped and uncapped mRNAs) followed by stainning with methylene blue. (B) The effect of divalent cations on RSV En nuclease activity. Purified RSV En and substrates were incubated with 1 mM $MnCl_2$, $MgCl_2$, or $CaCl_2$ at 37℃ for 30 min.

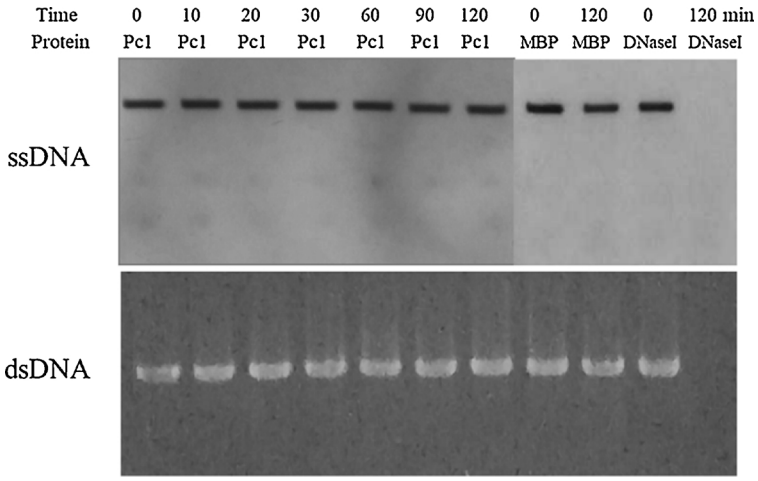

Fig. 4. RSV En Endonuclease activity on DNA. Purified RSV En was incubated with substrates at 37℃ in the presence of 1 mM Mn^{2+}. The incubation was stopped by adding loading buffer after 10, 20, 30, 60, 90 and 120 min. MBP and DNaseI were used as a negative and positive control, respectively. The ssDNA reaction products were assessed by electrophoresis on a 15% polyacylamide gel containing 8 M urea and stained with methylene blue. The dsDNA reaction products were separated by electrophoresis on a 1% agarose gel and stained with ethidium bromide.

was digested by En (Fig. 4), showing that the enzyme is RNA specific. Together, these results demonstrated that RSV En was a Mn^{2+}-dependent RNA-specific nuclease.

To identify the residues responsible for nuclease activity of Pc1, we generated a series of alanine point mutants of putative key residues. Based on previous sequence alignment and structural comparison of endonucleases of bunyaviruses (Reguera et al., 2010; Rothenberger et al., 2016; Reguera et al., 2016), we assumed that RSV Pc1 residue H535, D547, D567, E585, T587 and K604 corresponded to H34, D52, D79, D92, K94 and K108 of LACV L-protein and H36, E54, D97, E110, T112 and K124 of Hantaan virus (HTNV) L-protein, respectively. For LACV, H34, D52, D79, D92 and K94 have been previously identified as the key residues of active site of endonuclease (Reguera et al., 2010). Therefore, we mutated the corresponding five residues of RSV Pc1 (H535, D547, D567, E585 and T587) to alanine. In addition, K124 of HTNV was reported to be an essential catalytic residue for endonuclease activity (Reguera et al., 2016). So, we also replaced the corresponding K604 in RSV Pc1 with alanine. Six En mutants (H535A, D547A, D567A, E585A, T587A and K604A) were generated via a classical two-step PCR mutagenesis approach as described previously (Hass et al., 2008) and expressed in *E. coli* followed by purification through amylose affinity chromatography. SDS-PAGE showed that all mutant proteins were purified with high purity except D567 A (Fig. 5A). The in vitro nuclease activities of these mutant proteins were assayed with 1 mM Mn^{2+} and

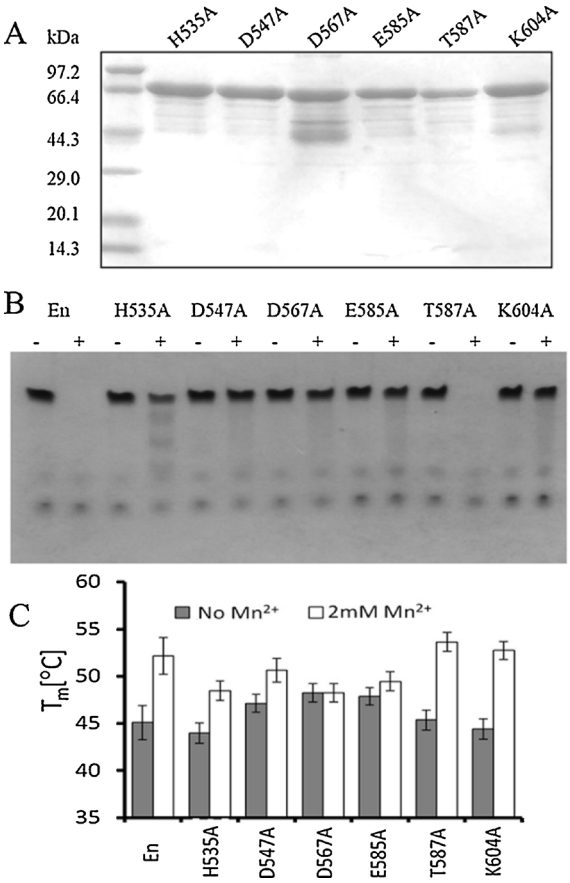

Fig. 5. Endonuclease activity assays of RSV En mutants. (A) Expression and purification of RSV En mutants in *E.coli.* (B) In vitro nuclease activity of RSV En and its mutants. U-rich ssRNA was incubated with 1 mM Mn^{2+} in the presence (+) or absence (-) of RSV En or mutants. The digestion was performed at 37°C for 30 min and then the degradation products were assayed as described in Fig. 3. (C) Thermal stability of RSV En mutants. Protein stability of wild-type and mutant En proteins were analyzed in thermofluor experiment in the absence and presence of 2 mM Mn^{2+}. The data represented mean and standard deviation of three independent experiments.

U-rich RNA in parallel with the wild-type En. The mutations D547A, D567A, E585A and K604A led to elimination of nuclease activity, the mutation H535A reduced but not eliminated activity, and the mutation T587A had no effect on activity (Fig. 5B). The above experimental results demonstrated that D547, D567, E585 and K604 were essential for enzymatic activity. His535 was also important for activity. In contrast, Thr587 was not related to activity. To determine the role of the mutated residues in ion binding, we also tested the influence of mutation on protein stability without and with 2 mM Mn^{2+} by thermofluor assay as described (Ericsson et al., 2006). Addition of Mn^{2+} significantly enhanced the thermal stability of wild-type En (Fig. 5C). The mutant T587A and K604A showed similar Mn^{2+} stabilization effect to that of wild-type En (Fig. 5C). The mutant H535A reduced the Mn^{2+} stabilization effect (Fig. 5C). The mutant D547A and E585A impaired and D567A completely lost the Mn^{2+} stabilization effect (Fig. 5C). These results suggested that H535, D547, D567 and E585 were engaged in ion coordination but T587 and K604 were not.

Taken together, the biochemical data here provided experimental evidence for the nuclease activity of RSV Pc1. The enzymatic activity was dependent on Mn^{2+} rather than Mg^{2+}, which showed very similar ion dependence to the endonucleases of the order *Bunyavirales* and family *Orthomyxoviridae* despite observation of weaker activity with Mg^{2+} for influenza virus PA (Morin et al., 2010; Reguera et al., 2010; Wallat et al., 2014; Reguera et al., 2016). Substrate specificity of RSV En, active on RNA but not DNA, resembled that of ANDV endonuclease (Fernández-García et al., 2016) but differed from that of influenza virus and LACV endonucleases (Reguera et al., 2010). In latter two viruses, the endonuclease was able to degrade both RNA and DNA. The nuclease activities of these endonucleases on RNA were consistent with their functions of cleaving host mRNAs during the process of cap-snatching.

Based on the results from mutational analysis of the in vitro nuclease activity and thermal stability of RSV En and the comparison the endonuclease of RSV with those of LACV (family *Peribunyaviridae*) and HTNV (family *Hantaviridae*) within the same *Bunyavirales* order, we could speculate the role of each key residue in enzymatic activity. As the residue D52, D79 and D92 of LACV L protein or E54, D97 and E110 of HTNV L-protein, RSV Pc1 residue D547, D567 and E585 possibly contributed to the canonical binding of metal ions. RSV Pc1 residue K604, equivalent to K94 (LACV) and K124 (HTNV), was supposed to function as a key catalytic residue in the active sites. Remarkably, mutation of H535 still retained some enzymatic activity although the activity was relatively low compared with the wild-type En, which was different from the endonucleases of other bunyaviruses with mutation of His completely abrogating the endonucease activity. Thermal stability experiment revealed that H535 was involved in ion binding but not as important as other ion binding residues D547, D567 and E585. The discrepancy was possibly due to their structural difference, which needed to be clarified in future.

In summary, we have characterized an endonuclease in RSV Pc1. Consistent with its function in cap-snatching, the enzyme has a RNA-specific nuclease activity in the presence of Mn^{2+}. Unlike most other characterized cap-snatching endonucleases of bunyaviruses, RSV endonuclease domain is not directly located at the N-terminus of protein. The catalytic properties and key catalytic residues of RSV endonuclease were similar but not identical to other viral endonucleases of the order *Bunyavirales*, suggesting that RSV endonuclease may have some distinct catalytic characteristics which may help to develop specific antiviral against RSV.

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