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cis-Acting core RNA elements required for negative-strand RNA synthesis and cap-independent translation are separated in the 3′-untranslated region of *Red clover necrotic mosaic virus* RNA1

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Abstract

The genome of *Red clover necrotic mosaic virus* (RCNMV) is positive-sense and divided into RNA1 and RNA2. RNA1 has a translation enhancer element (3′ TE-DR1) in the 3′ untranslated region (UTR) that substitutes for a 5′ cap. In this study, we determined the regions required for cap-independent translation and RNA synthesis in the 3′ UTR of RNA1 using a cell-free extract of evacuolated BY-2 protoplasts (BYL) and by an assay in BY-2 protoplasts. The use of capped viral RNA transcripts in the BYL system allowed us to distinguish the effects of introduced mutations on cap-independent translation and negative-strand RNA synthesis of RNA1. We found that the core RNA element of 3′ TE-DR1 essential for cap-independent translation of RNA1 is dispensable for negative-strand RNA synthesis. Thus, *cis*-acting RNA elements essential for cap-independent translation are separated from those required for negative-strand RNA synthesis in the 3′ UTR of RCNMV RNA1.

Keywords: Cap-independent translation; 3′ untranslated region; 3′ translational element; Negative-strand RNA synthesis; In vitro replication assay; Stem–loop structure; *Tombusviridae*; *Dianthovirus*; Cap-structure; RNA replication

Introduction

To proliferate in the host cell, positive-sense RNA viruses translate replicase proteins and synthesize genomic RNAs through negative-strand intermediates using these viral replicase proteins and host factors. In viral RNA replication, the 3′ untranslated region (UTR) of its genome is essential for the initiation of negative-strand RNA synthesis. Furthermore, the 3′ UTR also plays an essential role in translation in many RNA viruses (Dreher, 1999; Edgil and Harris, 2006; Kneller et al., 2006). In several RNA viruses lacking the cap structure at the 5′ end, translation-enhancer elements in the 3′ UTR of genomic RNAs substitute for the cap structure to recruit ribosomes (Kneller et al., 2006). Thus, the 3′ UTR of viral RNAs is a key player in translation, replication and translation–replication regulations in many RNA viruses.

To investigate the translation and replication mechanisms of RNA viruses, we used *Red clover necrotic mosaic virus* (RCNMV), a member of the genus *Dianthovirus* in the family *Tombusviridae*. Its genome consists of two positive-sense single-stranded RNAs, RNA1 and RNA2, which have no 5′ cap structure and no poly(A) tail at the 3′ end. RNA1 encodes putative replicase components, a 27-kDa protein (p27) and its N-terminal overlapping frameshift product, an 88-kDa protein (p88) (Kim and Lommel, 1994; Xiong et al., 1993b), which has an RNA-dependent RNA polymerase motif (Koonin, 1991). RNA1 also encodes a 37-kDa coat protein (CP), which is expressed from a subgenomic RNA (sgRNA) (Zavriev et al., 1996). Transcription of the CP sgRNA requires an intermolecular interaction between RNA1 and RNA2 (Sit et al., 1998). RNA2 is a monocistronic RNA, which encodes a 35-kDa movement protein (MP) that is required for viral cell-to-cell movement in plants (Lommel et al., 1988; Xiong et al., 1993a).

All these viral proteins are translated in cap-independent mechanisms (Mizumoto et al., 2003, 2006), in contrast to eukaryotic cellular mRNAs whose translation is dependent on

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cap structure and poly(A) tail. Interestingly, the cap-independent translation mechanism differs between RNA1 and RNA2 (Mizumoto et al., 2006). RNA1 possesses a translation enhancer element (3′ TE-DR1) in the 3′ UTR that replaces the function of the cap structure (Mizumoto et al., 2003). Such translation enhancer elements in the 3′ UTR have been detected in viruses in the families *Tombusviridae* and *Luteoviridae* (Kneller et al., 2006). The translation enhancer elements of *Tobacco necrosis virus* (TNV) in the genus *Necrovirus* and *Barley yellow dwarf virus* (BYDV) in the genus *Luteovirus* have a conserved 17 nt core sequence (Meulewaeter et al., 2004; Shen and Miller, 2004; Wang et al., 1997) that is also conserved in the 3′ TE-DR1 in RCNMV RNA1 (Mizumoto et al., 2003). On the other hand, RNA2 does not have such translation-enhancer elements, and the cap-independent translation activity of RNA2 is linked to RNA2 replication (Mizumoto et al., 2006). This indicates that RNA elements necessary for RNA replication also are essential for cap-independent translation in RNA2. RNA elements required for the replication of RNA2 are present in the 5′ and 3′ UTRs (Turner and Buck, 1999) and in the MP ORF of RNA2 (Tatsuta et al., 2005). A 3′ proximal stem–loop (SL) structure in the 3′ UTR of RNA1 appears important for the replication of RNA1, because the SL is conserved between RNA1 and RNA2, and is essential for negative-strand RNA synthesis in RNA2 (Takeda et al., 2005; Turner and Buck, 1999). Thus, single nucleotide substitution in the stem of the SL of RNA1 is sufficient to alter the temperature-sensitive phenotype in RNA accumulation in protoplasts (Mizumoto et al., 2002). Together, these data suggest that the 3′ UTR of RCNMV RNA1 plays a central role in cap-independent translation, in RNA replication and in their regulation.

To obtain insights into regulation between translation and replication in RCNMV RNA1, it is necessary to determine regions required for cap-independent translation, negative-strand RNA synthesis or both in the 3′ UTR of RNA1. However, it is difficult to analyze RNA replication independently of translation, because any mutations affecting cap-independent translation will also affect RNA replication. Furthermore, p27 and p88 do not effectively function in *trans* for the replication of RNA1 mutants that encode one of these proteins, or no viral proteins (Takeda et al., 2005) (K. Okamoto, and T. Okuno, unpublished data). To overcome these problems, we used a cell-free extract in vitro assay system (BYL) that is prepared from evacuolated BY-2 protoplasts (Komoda et al., 2004) in addition to an assay in BY-2 protoplasts. The BYL system has several advantages over the protoplast system. BYL reflects the cap-independent translational activity of reporter luciferase (Luc) mRNAs with a series of mutations in the 3′ UTR of RNA1 in cowpea protoplasts (Mizumoto et al., 2003, 2006). In BYL, negative-strand RNAs are easily detected following translation of viral replication proteins, even if input transcripts are incompetent in positive-strand RNA synthesis (H. Iwakawa and T. Okuno, unpublished results; this paper). Furthermore, the use of capped viral RNA transcripts in the BYL system allowed us to distinguish the effects of introduced mutations on cap-independent translation and RNA synthesis.

Our results indicated that the translation enhancer core RNA element essential for cap-independent translation of RNA1 was not required for negative-strand RNA synthesis, and that two SL structures and nucleotide sequences between the two SLs in the 3′ proximal region of RNA1, which are conserved among RNA1 and RNA2 and for all members in the genus *Dianthovirus*, are essential for the negative-strand RNA synthesis of RCNMV RNA1.

Results

Our previous study indicated that a 3′ TE-DR1 including SL1 was essential for the cap-independent translation of RCNMV RNA1 in cowpea protoplasts using reporter Luc mRNAs (Mizumoto et al., 2003). However, the study leaves open questions on roles of regions other than 3′ TE-DR1 in the 3′ UTR for cap-independent translation in full-length RNA1 and for RNA replication. To address these questions, first we determined regions required for the efficient accumulation of p27 and replication of RNA1 in BY-2 protoplasts using RNA1 deletion mutants with deletions in the 3′ UTR (d3′SLA, d3′SLB, d3′SLC, d3′SLD, d3′SLE, d3′SLF, and d3′TE) (Figs. 1A and B). d3′TE lacked the central region of 3′ TE-DR1 (between nucleotides 3607 and 3719, designated 3′ TE-DR1-c) was deleted. These mutants were designed based on RNA secondary structures predicted for the 3′ UTR of RCNMV (Australian strain) RNA1 in concert with the 3′ UTR of another RCNMV RNA1 (Canadian strain) by the computer algorithm Dynalign (Mathews and Turner, 2002) (Fig. 1A). BY-2 protoplasts were inoculated with these RNA1 mutants, and accumulations of p27 and positive-strand and negative-strand RNA1s were analyzed by western blotting using anti-p27 antibody and northern blotting, respectively. p27 was detected upon inoculation with wt RNA1 or d3′SLA, but not after inoculation with d3′SLB, d3′SLC, d3′SLD, d3′SLE, d3′SLF, or d3′TE (Fig. 1C). Positive-strand and negative-strand RNA1s were detected following inoculation with wt RNA1 or d3′SLA (Fig. 1C). These results suggested that RNA1 mutants other than d3′SLA were incompetent in either translation or replication.

Next, we analyzed the translational activity of the same mutants using a BYL in vitro translation assay system, because this allows one to assess the cap-independent translational activity of full length RNA1 mutants, even when the mutants are incompetent in replication (Mizumoto et al., 2006). Uncapped RNA1 mutants were incubated in BYL reaction mixture at 17 °C for 2 h. Western blot analysis showed that wild type RNA1, d3′SLA, d3′SLB, d3′SLD, d3′SLE and d3′SLF accumulated p27, whereas d3′SLC and d3′TE did not (Fig. 1D). To investigate the effects of deletions on the stability of RNA, RNA degradation profiles of capped or uncapped RNA1 mutants were monitored in the BYL assay by northern blotting. The degradation profiles of uncapped d3′SLC and d3′TE were not significantly different from that of wild type RNA1 (Fig. 1E). Thus, regions other than the SLC that includes 3′ TE-DR1 are not essential for the cap-independent translation of full-length RNA1 in BYL, confirming our previous results obtained by using reporter Luc mRNAs (Mizumoto et al., 2003). These results also suggested that