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Short communication

Replication of cucumber mosaic virus RNA 1 in *cis* requires functional helicase-like motifs of the 1a protein

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ABSTRACT

The cucumber mosaic virus (CMV) encoded 1a protein contains an NTPase/helicase-like domain. To investigate whether various helicase motifs were required for efficient replication and to establish whether CMV RNA 1 could be replicated efficiently in *cis*, we constructed deletion mutations in helicase motifs I, III and VI and analyzed their effects on CMV RNA replication in tobacco. CMV replication was not detectable for any of the three helicase mutants, indicating that the helicase domain is crucial for efficient CMV replication. Both the wild-type and mutant 1a proteins could be detected at similar levels after transient expression in infiltrated tissues, indicating that the helicase-motif mutations did not affect the stability of the proteins. Co-inoculation tests with various mutant combinations did not result in complementation. In protoplasts derived from CMV RNA 1-transgenic tobacco, which supported replication of CMV RNAs 2 and 3, the RNA 1 helicase mutants were not replicated detectably in *trans*, but also did not interfere with the replication of the genomic RNAs, indicating that the conserved helicase motifs of the 1a protein are required in *cis* for the effective accumulation of RNA 1.

The genomes of positive-strand RNA viruses encode functionally similar proteins involved in virus replication. In many cases, such as in members of the *Bromoviridae*, different proteins are associated with the RNA-dependent RNA polymerase activity (2a protein) and with functions such as unwinding (helicase-like) and capping (1a protein) (Koonin and Dolja, 1993; Kadaré and Haenni, 1997). These 1a proteins have similarities to known helicases that bind their target RNA and catalyze RNA duplex unwinding using hydrolysis of ATP as an energy source (Caruthers and McKay, 2002). Based on sequence comparisons, the *Bromoviridae* 1a proteins have a C-terminal superfamily I NTPase/helicase-like domain, containing the six helicase signature motifs, denoted I to VI (Habili and Symons, 1989; Kadaré and Haenni, 1997). These include putative NTP-binding regions (motifs I and II), a putative ATPase activity (motif III) and possibly RNA binding activity (motif VI). It has been shown that mutations in specific helicase motifs of either alfalfa mosaic virus (AMV; motifs I, III and VI) (Vlot et al., 2003), or brome mosaic virus (BMV; motifs I and II to VI) (Wang et al., 2005) strongly reduced accumulation of the genomic RNAs. In addition, in-*trans* replication of AMV RNA 1 and BMV RNA 1 with mutations in the 1a protein helicase domain either did not occur detectably or occurred at only a lower efficiency in the presence of the wild-type (wt) 1a protein expressed from either an agroinfiltrated plasmid, or the host genome. In some cases, the mutant 1a proteins also interfered with the replication of other viral RNAs in *trans* (Van Rossum et al., 1996; Vlot et al., 2003; Wang et al., 2005; Yi and Kao, 2008). In the case of cucumber mosaic virus (CMV), a point mutation in helicase motif I prevented the 1a protein from functioning in (−) CMV RNA synthesis (Seo et al., 2009). However, it has not been determined whether the helicase motifs III or VI also are required for CMV replication, whether CMV RNA 1 helicase domain mutants dysfunctional for replication of other CMV RNAs in *trans* can themselves be replicated in *cis*, nor whether replication-defective 1a proteins affect replication of other CMV RNAs in *trans*. These properties were examined here.

Single point mutations in CMV replication-associated genes sometimes are unstable and revert to wt sequences (Du et al., 2008; Seo et al., 2009). Thus, we deleted the sequences from helicase motifs I (nucleotides 2219–2270), III (nucleotides 2507–2542), and VI (nucleotides 2975–3022) by PCR-mediated mutagenesis (Sambrook et al., 1989), using the primer pairs

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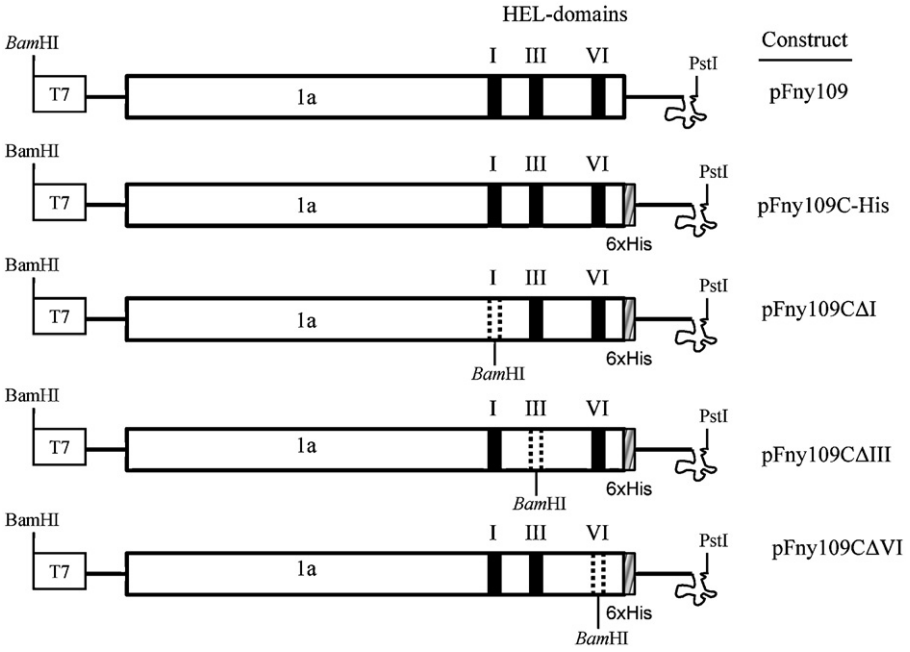

Fig. 1. Schematic representation of the cDNA constructs of CMV RNA 1. The conserved helicase-like motifs (HEL-motifs) I, III, and VI in CMV 1a protein used for constructions of deletion-mutant cDNA clones are indicated as black boxes. The sequences encoding helicase-like motifs I, III, and VI, deleted in mutants FΔI, FΔIII, and FΔVI, respectively, are indicated as dashed lines. The *Bam*HI restriction sites that were introduced into the deletions are indicated. The long rectangle represents the coding sequence of 1a protein. The 5′ and 3′ noncoding regions of CMV RNA 1 are shown as black lines. The 3′ tRNA-like structure of CMV RNA 1 is indicated by a squiggly line. The T7 RNA promoter and *Pst*I restriction sites, used for synthesis of full-length transcript RNA 1 *in vitro* and linearization of the plasmid, respectively, are indicated. The 3′ end of the gene for the 1a protein contains a sequence that encodes six histidines (shaded boxes).

given in Supplementary Table 1 and the strategy described in Supplementary Fig. 1. These primers contained a *Bam*HI site inserted in place of the deleted helicase motif to facilitate the joining of PCR fragments for ligation into pUC18. We also used the biologically active cDNA of CMV, Fny-CMV RNA 1 (pFny109C-His), modified by addition of sequences that encode hexa-histidine residues at the C-terminus of the la protein, as the template (Fig. 1), since this had no effect on the replication of CMV RNAs (Gal-On et al., 2000).

The biological need for the helicase domain in viral RNA replication was ascertained as follows. RNAs were transcribed from full-length cDNA clones of pFny109, pFny209, and pFny309, representing Fny-CMV RNAs 1, 2, and 3, respectively (Rizzo and Palukaitis, 1990), as well as from the three 1a-helicase mutant constructs, pFny109CΔI, pFny109CΔIII and pFny109CΔVI (Fig. 1), all as described previously (Choi et al., 2005). Transcript mixtures, consisting of wt RNAs 2 and 3, as well as either wt or mutant RNA 1, were concentrated by ethanol precipitation prior to use in mechanical inoculations on aluminum oxide-dusted leaves of either *Nicotiana benthamiana* or *N. tabacum* cv. Samsun NN (tobacco NN). The accumulation of viral RNA in the inoculated leaves was analyzed at 3 days post-inoculation (dpi) by northern blot hybridization of total RNAs extracted from the inoculated plants, as described previously (Canto and Palukaitis, 2001). Accumulation of (+) CMV RNAs was not detectable in total RNAs extracted from either *N. benthamiana* leaves or tobacco NN leaves inoculated with mixtures of transcripts containing any RNA 1 helicase mutant and wt CMV RNAs 2 and 3. We also did not detect any (−) CMV RNAs by northern blot hybridization (data not shown). By contrast, a mixture of transcripts containing wt CMV RNAs 1, 2 and 3 showed high accumulation of (+) CMV RNAs in the inoculated leaves of both plant species (Fig. 2A).

RT-PCR analysis also was performed on the above nucleic acid samples using primer pairs specific to CMV RNA 3 (listed in Supplementary Table 1) and the synthesis conditions described previously (Choi et al., 1999), to ascertain if any of the 1a-helicase mutants might be replicating below the detection limit of northern blot hybridization. However, no RT-PCR products were detectable using total RNA extracts derived from plants inoculated with wt CMV RNAs 2 and 3 plus any of the 1a-helicase mutants, while RT-PCR products were detectable in total RNAs extracted from plants inoculated with wt CMV RNAs 1, 2, and 3 (Fig. 2B). Thus, it appears that deletion of helicase motifs I, III or VI inhibited the encoded 1a protein from functioning in *trans* replication of the CMV RNAs 2 and 3. Since the 1a protein was shown to be essential for (−) CMV RNA synthesis but was not required for (+) CMV RNA synthesis (Seo et al., 2009), these results indicate that the various helicase motifs are essential for (−) CMV RNA synthesis, as was shown for AMV (Vlot et al., 2003) and BMV (Wang et al., 2005).

It is possible that the mutations in the helicase motifs of the 1a proteins affected the stability of the encoded proteins. Therefore, the above RNA 1 mutant DNAs were cloned into an *Agrobacterium*-expression plasmid pC-TAPa, transformed into *Agrobacterium tumefaciens* strain GV3101 and agroinfiltrated into *N. benthamiana* leaves as described previously (Rubio et al., 2005). Three days after infiltration, proteins were extracted from agroinfiltrated areas and subjected to western blot analysis using an antiserum to the cMyc-tag, also as described previously (Rubio et al., 2005). Both the wt and mutant 1a proteins could be detected by this assay and the levels of accumulation were similar for the wt and three 1a-helicase mutant proteins, comparable to the coilin protein control (Fig. 3). Thus, it is unlikely that the failure to detect viral RNA accumulation was due to instability of the mutant 1a proteins.

To assess whether complementation could occur among helicase mutants of the 1a protein, leaves of *N. benthamiana* were inoculated with a mixture of transcripts containing CMV RNAs 1 harboring any two different 1a-helicase mutants, plus wt Fny-CMV RNAs 2 and 3. RT-PCR results showed that the cells containing various helicase mutants did not support the detectable synthesis of