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Infectious cDNA transcripts of *Maize necrotic streak virus*: Infectivity and translational characteristics

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Abstract

Maize necrotic streak virus (MNeSV) is a unique member of the family Tombusviridae that is not infectious by leaf rub inoculation and has a coat protein lacking the protruding domain of aureusviruses, carmoviruses, and tombusviruses (Louie et al., Plant Dis. 84, 1133–1139, 2000). Completion of the MNeSV sequence indicated a genome of 4094 nt. RNA blot and primer extension analysis identified subgenomic RNAs of 1607 and 781 nt. RNA and protein sequence comparisons and RNA secondary structure predictions support the classification of MNeSV as the first monocot-infecting tombusvirus, the smallest tombusvirus yet reported. Uncapped transcripts from cDNAs were infectious in maize (*Zea mays* L.) protoplasts and plants. Translation of genomic and subgenomic RNA transcripts in wheat germ extracts indicated that MNeSV has a 3′ cap-independent translational enhancer (3′CITE) located within the 3′ 156 nt. The sequence, predicted structure, and the ability to function in vitro differentiate the MNeSV 3′CITE from that of *Tomato bushy stunt virus*.

Keywords: Tombusvirus; Infectious transcripts; *Maize necrotic streak virus*; MNeSV; Cap-independent translation; Translational enhancer

Introduction

Maize necrotic streak virus (MNeSV) is a monopartite single-stranded positive sense RNA virus, and partial genome sequence analysis showed it to be related to members of the family Tombusviridae (Louie et al., 2000). The genome organization of MNeSV was found to be similar to that of tombusviruses and aureusviruses with five open reading frames (ORFs). Nonstructural proteins encoded by MNeSV were most similar to tombusviruses, even though the estimated size (~4.3 kb) of virion RNA (vRNA) was closer to that of aureusviruses. Analysis of the predicted 27.4 kDa coat protein (CP) of MNeSV indicated that it did not contain the protruding domain found on tombusvirus and aureusvirus CPs and was most closely related to CPs of necroviruses. The virion size of 32 nm was closer to that of tombusviruses (32–35 nm) (Lommel et al., 2005b) than necroviruses (28 nm) (Lommel et al., 2005a). Similarly to tombusviruses, no insect vector of MNeSV was identified among eight insect species tested (Louie et al., 2000). A characteristic of MNeSV that differentiated it from previously identified tombusviruses was that the virus could not be transmitted by leaf rub inoculation (Louie et al., 2000). However, MNeSV readily infected maize (*Zea mays* L.) via vascular puncture inoculation (VPI) of seeds.

Several species of dicot-infecting tombusviruses have been used to study various aspects of the virus life cycle: replication in vivo (Fabian et al., 2003; Ray et al., 2003; Wu et al., 2001), in vitro (Nagy and Pogany, 2000; Panavas et al., 2002), and in yeast (Panavas and Nagy, 2003; Pantaleo et al., 2003); recombination and production of defective interfering-RNAs (DI-RNAs) (Burgyan et al., 1991; Reade et al., 1999; Scholthof, K. et al., 1995); transcription (Choi and White, 2002; Choi et al., 2001; Lin and White, 2004); in vivo translation (Fabian and White, 2004; Wu and White, 1999); cell-to-cell and long-distance movement (Chu et al., 2000; Scholthof, H. et al., 1995); initiation and suppression of gene silencing (Havelda et al.,

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2003; Qiu et al., 2002); and fungal transmission (McLean et al., 1994). The similarity of MNeSV to tombusviruses suggested that it might be the first monocot-infecting tombusvirus and could thus provide a very useful tool to compare aspects of the tombusviral life cycle in a monocot system.

The genomes of viruses in the family Tombusviridae are uncapped and do not contain poly(A) tails, two structures required for efficient translation of most eukaryotic mRNAs. For plant mRNAs, the ^{m}7GpppN cap is bound by initiation factor eIF4F or eIFiso4F, the poly(A) tail is coated by poly(A) binding protein, and both proteins bind to additional initiation factors to form a circular structure which efficiently recruits the 40S ribosome subunit (Kawaguchi and Bailey-Serres, 2002). Many viruses use alternative 5′ and/or 3′ structures to efficiently translate their mRNAs. In the family Tombusviridae, 3′ translational enhancers (3′TEs) have been previously identified in *Tomato bushy stunt virus-C* (TBSV-C) (Wu and White, 1999), the necroviruses *Tobacco necrosis virus-A* (TNV-A) and TNV-D (Meulewaeter et al., 2004; Shen and Miller, 2004) and their satellite virus STNV (Danthinne et al., 1993; Timmer et al., 1993), the dianthovirus *Red clover necrotic mosaic virus* (Mizumoto et al., 2003), and the carmoviruses *Turnip crinkle virus* (Qu and Morris, 2000) and *Hibiscus chlorotic ringspot virus* (Koh et al., 2002). TBSV does not naturally infect cereals, and its 3′ cap-independent translational enhancer (3′CITE) is active in cucumber protoplasts but not in wheat germ extract (WGE) (Wu and White, 1999). As a monocot-infecting tombusvirus, we hypothesized that MNeSV might provide a tool for analyzing cap-independent translation of a tombusvirus in WGE, a system used for the well-studied 3′TE of *Barley yellow dwarf virus*-PAV (BYDV-PAV) (Allen et al., 1999) and other viruses.

In this paper, we report the completed sequence of MNeSV and the construction of infectious transcript cDNAs. We also mapped the two subgenomic RNAs (sgRNAs) and identified predicted secondary structures in MNeSV genomic RNA (gRNA) similar to those found for dicot tombusviruses. Lastly, we have identified a cap-independent translational enhancer in the 3′ untranslated region (UTR) of MNeSV that functions on gRNA and sgRNAs in WGE. These results indicate that MNeSV should be classified as a tombusvirus.

Results

MNeSV genome sequence and secondary structure

Analysis of the initial sequence data for MNeSV suggested that about 10% of the viral sequence was missing based on the vRNA size estimate. Two approaches were used to complete the sequence of the 5′ end of the MNeSV genome. First, cDNAs synthesized with RNA ligase-mediated rapid amplification of cDNA ends (RLM-RACE) of vRNA that was not pretreated with calf intestinal phosphatase (CIP) or tobacco acid pyrophosphatase (TAP) were cloned into a plasmid vector and sequenced (Table 1). No cDNA could be amplified from vRNA that was pretreated with CIP followed by TAP before the RNA ligation reaction (data not shown), indicating that the vRNA was uncapped. Four cDNAs with the same sequence and one with a deletion of at least two A's corresponding to the 3′ end of the RNA oligomer, which terminated with GAAA, were identified (Table 1). These data suggested the RNA oligomer was partially deteriorated. Using an alternative strategy for RACE, cDNA synthesized from vRNA was C-tailed then used for PCR. The sequence of these cloned PCR products indicated the 5′ viral sequence was AGAUAU (Table 1). This sequence was consistent with results of primer extension analysis which produced cDNAs one base longer than the GATAT found in RLM-RACE-derived clones (Fig. 1). Thus, it is likely that the RNA primer or the vRNA was degraded prior to ligation in the clones derived from RLM-RACE and that the 5′ sequence of the MNeSV RNA is AGAUAU. This sequence is consistent with that of many tombusviruses (White and Nagy, 2004). The sequences of the 5′ RACE products indicated that there were 28 additional nt at the 5′ end of the viral genome relative to the previously published sequence (Louie et al., 2000). In addition, sequences of the RACE products indicated that the residue at nt 88 of the full-length sequence was a C rather than the U reported earlier.

Table 1
Sequences of the 5′ end of the MNeSV vRNA determined using RACE

Method [a]	# clones [b]	Sequence [c]
RLM-RACE	4	atgaaaGATAT
RLM-RACE	1	atga GATAT
5′ RACE	2	gggggAGATAT
Tombusvirus consensus [d]		AGAAAU

[a] The sequences of cDNAs were obtained using the RLM-RACE kit (Ambion) with untreated vRNA or a 5′ RACE kit (Invitrogen) using total RNA.
[b] The number of cDNAs showing the indicated sequence.
[c] Sequences corresponding to viral RNA are indicated in uppercase letters, and those corresponding to the RNA oligomer or complement of the C-tail are indicated in lower case letters.
[d] The dicot tombusvirus consensus vRNA 5′ terminal sequence (White and Nagy, 2004).

The initial MNeSV sequence data terminated within ORF5, indicating that some of the coding region and the 3′UTR were missing. The 3′ end of the MNeSV genome was obtained using anchored cDNA cloning (Weng and Xiong, 1995) with an upstream primer corresponding to nt 3623–3647 of the previously published MNeSV sequence (nt 3651–3675 of the complete sequence, GenBank accession number AF266518). Three cDNAs were sequenced, and two positions showed transitions at nt 3909 (A/G) or 3988 (C/T). Analysis of the resulting data indicated that the previously published partial sequence contained a duplicated fragment (nt 2188–2290 of the viral genome) inserted after nt 3832. After accounting for the duplication, 258 nt of additional sequence was identified in the anchored cDNA clones. Thus, the complete sequence of MNeSV is 4094 nt which is about 660 nt smaller than most dicot tombusviruses and is 482 nt smaller than *Cucumber Bulgarian latent virus* (CBLV GenBank accession number AY163842).

With the sequence completed, we reevaluated the positions and sizes of the ORFs, the similarity of encoded proteins and RNA, and predicted RNA secondary structures for comparison