Virus Research 222 (2016) 71–79

Contents lists available at ScienceDirect

Virus Research

journal homepage: www.elsevier.com/locate/virusres

Analysis of gene functions in *Maize chlorotic mottle virus*

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ARTICLE INFO

Article history:
Received 9 January 2016
Received in revised form 11 April 2016
Accepted 18 April 2016
Available online 27 May 2016

Keywords:
Machlomovirus
Tombusviridae
RNA virus replication
Gene function
Viral movement
Overprinted gene

ABSTRACT

Gene functions of strains of *Maize chlorotic mottle virus,* which comprises the monotypic genus *Machlomovirus,* have not been previously identified. In this study mutagenesis of the seven genes encoded in maize chlorotic mottle virus (MCMV) showed that the genes with positional and sequence similarity to their homologs in viruses of related tombusvirid genera had similar functions. p50 and its readthrough protein p111 are the only proteins required for replication in maize protoplasts, and they function at a low level *in trans*. Two movement proteins, p7a and p7b, and coat protein, encoded on subgenomic RNA1, are required for cell-to-cell movement in maize, and p7a and p7b function *in trans*. A unique protein, p31, expressed as a readthrough extension of p7a, is required for efficient systemic infection. The 5′ proximal MCMV gene encodes a unique 32 kDa protein that is not required for replication or movement. Transcripts lacking p32 expression accumulate to about 1/3 the level of wild type transcripts in protoplasts and produce delayed, mild infections in maize plants. Additional studies on p32, p31 and the unique amino-terminal region of p50 are needed to further characterize the life cycle of this unique tombusvirid.

1. Introduction

Maize chlorotic mottle virus is the only member of the genus *Machlomovirus* in the family *Tombusviridae* (Rochon et al., 2011). The host range of maize chlorotic mottle virus (MCMV) is restricted to *Poaceae,* and MCMV is readily infectious by mechanical inoculation. MCMV is part of a synergistic disease called corn lethal necrosis (CLN) (Niblett and Claflin, 1978) or maize lethal necrosis (MLN) (Wangai et al., 2012) when it coinfects with a virus in the family *Potyviridae*. MCMV levels in MLN plants rise dramatically (Goldberg and Brakke, 1987; Scheets, 1998; Xia et al., 2016) as commonly seen with potyvirid synergisms.

The 5′ two-thirds of the 4437 nt viral RNA encode three proteins (Nutter et al., 1989). The first open reading frame (ORF) encodes a 32 kDa protein, p32 which is unique to MCMV (Fig. 1). p50 and its readthrough (rt) protein p111 are related to the highly conserved RNA dependent RNA polymerases (RdRps) encoded by other members of the family *Tombusviridae*. p50 is similar in size to the ORF1 product of *Panicum mosaic virus*, genus *Panicovirus,*(48 kDa) and two other panicoviruses (41–44 kDa) which are larger than the related ORF1-encoded proteins (23–37 kDa) of other tombusvirids. p50 has a unique amino-terminal sequence that coincides with most of the p32 ORF overlap (residues 1-283).

All ORFs in the 3′ third of the genome are expressed from subgenomic RNA1 (sgRNA1) (Scheets, 2000). The first ORF encodes a 7.2 kDa peptide (p7a) and suppression of its stop codon produces a 31 kDa protein, p31 (Scheets, 2000). The carboxy-terminus of p7a exhibits sequence similarity to movement protein 1 (MP1) found in tombusvirid genera that encode two or more small MPs (Rochon et al., 2011). A small ORF with no methionine codons (~8 kDa coding capacity) following the p7a ORF was later identified by comparison to carmovirus sequences (Riviere and Rochon, 1990), and the encoded peptide is most closely related to MP2 from panicoviruses (Scheets et al., 2015). MP1 and MP2 are required for cell-to-cell movement in viruses from four tombusvirid genera (Genoves et al., 2006; Hacker et al., 1992; Molnar et al., 1997; Pantaleo et al., 1999; Turina et al., 2000; Yuan et al., 2006) and an unassigned tombusvirid (Castaño et al., 2009; Scheets et al., 2015). The carboxy-terminal extension of the 31 kDa protein is unique. The second AUG of sgRNA1 begins the 25 kDa coat protein (CP) ORF (Scheets, 2000). A 337 nt noncoding RNA (sgRNA2) accumulates in MCMV infected protoplasts and plants (Scheets, 2000), although it is not known whether it is a true sgRNA or a structure-protected degradation product as determined for red clover necrotic mosaic virus (Iwakawa et al., 2008).

This project was undertaken to determine the functions of the MCMV-encoded proteins. As well as being noninfectious to dicots, MCMV is not amenable to expression of a reporter gene since attempts to replace MCMV coding regions or fuse a reporter to

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http://dx.doi.org/10.1016/j.virusres.2016.04.024
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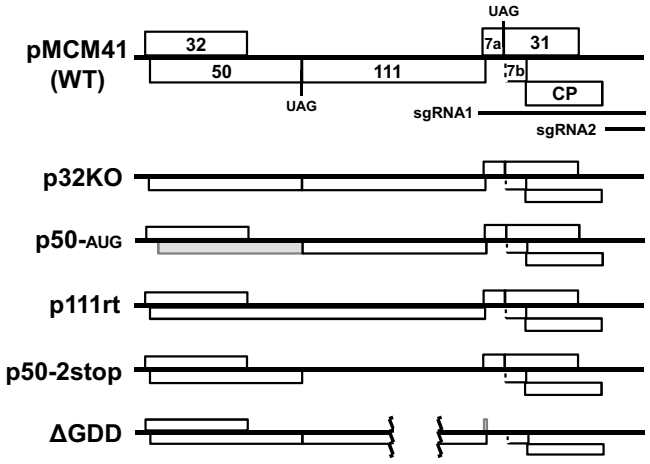

Fig. 1. Genome maps of WT and mutant plasmid inserts. Heavy lines represent genomic RNA, and boxes mark the coding regions and their relative reading frames. The locations of sgRNA1 and sgRNA2 are shown for WT. The sizes in kDa of the encoded proteins are shown, and the leaky stop codons are marked. Dashed lines mark a non-AUG start codon. Gray boxes denote smaller ORFs produced by start or stop codon mutations. The deleted region of ΔGDD is bordered by wavy lines.

the CP ORF were lethal (unpublished results). Complicating analyses, no good local lesion host for MCMV is available. Therefore, functions of all MCMV ORFs were analyzed by comparing the ability of mutant and wild type (WT) transcripts to replicate in maize protoplasts and to infect and spread in maize plants.

2. Materials and methods

2.1. Construction of mutant cDNAs and other plasmids used in the study

In vitro mutagenesis (Kunkel et al., 1987) of pMCM41 (Scheets et al., 1993) with oligonucleotides listed in Table S1 was used to make p32KO, p50-AUG, p111rt, and p50-2stop, while p7bMet, p7bKO, p7bpTer, and p7bQ12N were made using a Gene Editor kit (Promega Corp., Madison WI). Construction of pCP-AUG, p7apTer, p7art, and p7a/CP was described previously (Scheets, 2000). ΔGDD was serendipitously produced during p7apTer construction and is missing nt 2244–2675 with the same 3′ changes as p7apTer. Plasmids were selected by restriction digestion and/or sequencing (Scheets, 2000). Automated dideoxy sequencing was performed on an ABI instrument, and sequence data was analyzed using Sequencher (Gene Codes Corp., Ann Arbor, MI).

2.2. Black mexican sweet (BMS) maize protoplast inoculations

Protoplasts were isolated from BMS suspension cultures (Scheets, 2000; Scheets et al., 1993). For each experiment, equal aliquots ($1–3 \times 10^6$) of protoplasts were inoculated with equal amounts of mutant or pMCM41 transcript RNAs (7.5–15 μg) synthesized and purified as described previously (Scheets, 2000). For coinoculations of p111rt and p50-2stop transcripts, both RNAs were added at the concentration used in single inoculations. Samples were collected at 0, 24, and 48 h post-inoculation (hpi). CP detection by protein A sandwich-enzyme linked immunosorbent assay (PAS-ELISA) was described previously (Scheets, 2000). Dilutions of large scale purified MCMV virions (Scheets, 1998) quantified spectrophotometrically (6.7 cm^2 mg^{-1}) and assayed on the same 96-well plate were used to determine MCMV concentrations by linear regression. Concentrations at 48 hpi were converted to % WT. Total RNA isolation, electrophoresis, and phosphorimage detection was previously described (Scheets, 2000). Genomic RNA (gRNA) bands were quantitated from phosphorimage data and compared to WT levels for experiments that showed equivalent RNA sample loading.

2.3. Plant inoculations and visual symptom assessment

Maize (var. PH3168) was grown in greenhouses, and supplemental light was added for some experiments during months with short day lengths (October–February) to provide 13 h light. At the 3-leaf stage, plants were taken to the laboratory, dusted with carborundum and inoculated on the third leaf with 10 μl containing 8–10 μg transcript in 0.5% bentonite by three strokes with a gloved finger. Fresh gloves were used for each inoculum. For individual experiments the same amount of RNA was used/plant, and 3–6 seedlings/transcript were inoculated. For coinoculations, RNAs were mixed and added at the concentration used in single inoculations. Inoculated leaves were rinsed with deionized water 5–10 min post-inoculation. Plants were kept in a dark humid chamber overnight before returning them to the greenhouse. Barley seedlings (var. IAR/B/334-2) were grown and inoculated similarly except the second leaf was inoculated and light supplementation was for 14 h. Typically, MCMV systemic symptoms in PH3168 consist of a mild chlorotic mottle and appear first on leaf 4 or 5 about 4–8 days post-inoculation (dpi). The intensity of WT MCMV symptoms varies with growth conditions, with strongest symptoms produced by high light intensities and warm temperatures (28–31°C) (Scheets, 1998) such that after a week of cloudy weather in a greenhouse, previously observed mild symptoms may become almost invisible. In all 11 maize inoculation experiments, 100% of plants inoculated with WT transcripts were systemically infected.

2.4. CP detection in plants

For CP detection, inoculated leaves were sampled weekly (7 mm disc, 4–5 mg) (Scheets, 1998) beginning at 7 dpi. At 14 (or 13) dpi, 21 (or 20) dpi, and later time points, newest leaf samples were also collected. For some experiments, final samples were collected from all upper leaves. CP was quantified as for protoplast assays, and CP was converted to μg/sample before calculating the mean and standard error of the mean.

2.5. Northern blots of total RNA from plants and RNA dot blots of crude leaf extracts

Total RNA isolation and northern blots from water-washed inoculated leaves and newest leaves were done as in Scheets (1998). For some experiments duplicate 7 mm discs from inoculated leaves were washed by briefly vortexing in 0.2 ml 0.5% SDS, and the solution was discarded after 1 min. Washed inoculated leaf samples and upper leaf samples were stored at −80°C. The discs were ground in 0.2 ml GSCN Buffer and the crude extracts were prepared for blotting as described (Scheets, 2013). Thirty microliters (1/80 of sample) was loaded onto 96-well dot blots. Dilutions of similarly denatured full length transcripts of pMCM41 (5–50 ng/well) were used as standards.

2.6. Small-scale MCMV virion/vRNA isolations

Leaf tissue from each maize plant (0.15-0.4 g inoculated leaf and 0.34-0.5 g of upper leaf) was separately collected 20–30 dpi. Inoculated leaves were washed in 0.5% SDS, rinsed, and dried before all leaf samples were chopped and stored at −80°C. Mini-virion isolations were performed (Scheets, 1998) and stored at 4°C. MCMV vRNA was isolated and stored as dry RNA pellets at −20°C, then