Virus Research 163 (2012) 291–301

Contents lists available at SciVerse ScienceDirect

Virus Research

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

The ORF3-encoded proteins of vitiviruses GVA and GVB induce tubule-like and punctate structures during virus infection and localize to the plasmodesmata

Sabrina Haviv, Yoni Moskovitz, Munir Mawassi*

The S. Tolkowsky Laboratory, Department of Plant Pathology – The Virology Unit, Agricultural Research Organization, The Volcani Center, Bet Dagan 50250, Israel

ARTICLE INFO

Article history:
Received 16 August 2011
Received in revised form 11 October 2011
Accepted 18 October 2011
Available online 25 October 2011

Keywords:
Grapevine viruses
Vitiviruses
Movement protein
GFP
Protoplasts

ABSTRACT

The genomic RNA of vitiviruses contains 5 open reading frames (ORF). ORF3 encodes a protein to which the function of a movement protein (MP) was assigned, based on sequence homology with other viral proteins. The aim of the research described in this paper was to gain further insight in distribution profile of the ORF3 product encoded by the vitiviruses *Grapevine virus A* (GVA) and *Grapevine virus B* (GVB). Expression of the GVA MP–GFP fusion protein *via* the virus genome in *Nicotiana benthamiana* leaves resulted in the formation of irregular spots and fibrous network structures on the outermost periphery of epidermal cells. Expression of GVA MP–GFP and GVB MP–GFP was involved in the formation of the tubule-like and punctate structures on the periphery of *N. benthamiana* and *Vitis vinifera* protoplasts. Co-expression of the GVA MP–GFP and GVA MP–RFP in protoplasts resulted in co-localization of these proteins into the same punctate structures, indicating that the MP is not accumulated randomly onto the cell surface, but targeted to particular sites at the cell periphery, where punctate and tubule-like structures are likely formed. With the use of cytoskeleton and secretory pathway inhibitors, we showed that the cytoskeletal elements are not likely to be involved in targeting of the MP-GFP to the punctate cellular structures. In addition to MP, a functional coat protein was found to be essential for virus spread within inoculated leaves.

1. Introduction

Plant viruses generally encode one or more movement proteins (MPs) that are essential for their cell-to-cell movement and systemic spread. A variety of MPs were found to be associated with a number of subcellular structures, such as the endoplasmic reticulum (ER), cytoskeleton and plasmodesmata (Reichel et al., 1999; Lazarowitz and Beachy, 1999).

For plant viruses with an RNA genome, there are two main mechanisms for viral cell-to-cell movement that have been characterized. In the first, exemplified by *Tobacco mosaic virus* (TMV), the MP alters the size exclusion limit (SEL) of the plasmodesmata and enables cell-to-cell transport of a ribonucleoprotein complex composed of the viral RNA, the MP and additional viral replication complexes (Tomenius et al., 1987; Wolf et al., 1989). In this mechanism, the viral coat protein is dispensable for virus movement through the plasmodesmata. Apparently, movement of rod-shaped RNA viruses such as TMV and several icosahedral RNA viruses, as well as single-stranded DNA viruses is according to this mechanism (Fujiwara et al., 1993; Lazarowitz and Beachy, 1999).

The second mechanism, exemplified by the *Cowpea mosaic virus* (CPMV), is characterized by formation of tubular structures that traverse plasmodesmata through which virus particles move to neighboring cells. In this model the viral CP is required for virus movement through the plasmodesmata. The MP and the endomembrane secretion system are sufficient for tubule formation, while the cytoskeleton is involved in positioning of tubules relative to the plasmodesmata (Kasteel et al., 1993; Wellink et al., 1993; Laporte et al., 2003; Van Lent et al., 1990; Bertens et al., 2000). Tubules and peripheral punctate structures were found to be formed on CPMV-infected protoplasts in the absence of cell walls and plasmodesmata (Van Lent et al., 1991), and on protoplasts in which the MP is transiently expressed (Wellink et al., 1993). Such tubules in protoplasts have a similar ultra-structure to those in plant tissue and extend from the plasma membrane into the culture medium. The CPMV mechanism is typical for the icosahedral RNA viruses such as *comoviruses* (Van Lent et al., 1990), *nepoviruses* (Wieczorek and Sanfaçon, 1993; Ritzenthaler et al., 1995), *caulimoviruses* (Kitajima and Lauritis, 1969; Kasteel et al., 1996; Huang et al., 2001), *badnaviruses* (Cheng et al., 1998), *bromoviruses* (Kasteel et al., 1997; Van der Wel et al., 2000) and *tospoviruses* (Storms et al., 1995).

* Corresponding author at: The S. Tolkowsky Laboratory, Department of Virology, Agricultural Research Organization, The Volcani Center, Bet Dagan 50250, Israel. Tel.: +972 3 9683844; fax: +972 3 9604180.
E-mail address: mawassi@volcani.agri.gov.il (M. Mawassi).

0168-1702/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.virusres.2011.10.015

Grapevine virus A (GVA) is the type species of the genus *Vitivirus*, family *Betaflexiviridae*, which includes also *Grapevine virus B* (GVB), *Grapevine virus D* (GVD) (Martelli et al., 1997), *Grapevine virus E* (GVE) (Nakaune et al., 2008), *Heracleum latent virus* (HLV) (Bem and Murant, 1979) and *Mint virus* 2 (MV2) (Tzanetakis et al., 2007). GVA and GVB are closely associated with the rugose-wood disease complex of grapevine (Boscia et al., 1993, 1994; Garau et al., 1994). Their particles are elongated, filamentous and phloem-limited (Rosciglione et al., 1983; Tanne et al., 1989).

The complete genome sequences of GVA, GVB (Galiakparov et al., 1999; Minafra et al., 1994, 1997; Moskovitz et al., 2008) and GVE (Nakaune et al., 2008; Coetzee et al., 2010) have been reported. Only partial sequences are available for GVD (Abou-Ghanem et al., 1997). The genomes of GVA and GVB consist of a positive single-stranded RNA of ca. 7.5 kb, with a 3′ poly (A) tail. The genome of these viruses encodes five open reading frames (ORFs) (Galiakparov et al., 1999, 2003c; Saldarelli et al., 1996). ORF1 encodes a 194-kDa polypeptide with characteristics of replication-related proteins. ORF2 encodes a 20-kDa protein with no homology to other proteins. The protein encoded by the ORF4 is the coat protein. ORF5 encodes a protein that exhibits sequence similarities to small RNA binding proteins of various plant viruses (Galiakparov et al., 2003b) and that of GVA was found to possess suppression of RNA silencing activity (Chiba et al., 2006; Zhou et al., 2006). ORF3 of GVA and GVB encodes a 31-kDa (p31) or 36-kDa (p36) protein, respectively, to which the function of the movement protein was assigned, based only on amino acid similarity to putative movement proteins from the 30K super-family (Minafra et al., 1994). Intracellular distribution profile of the ORF3-encoded protein and its role in virus movement has not been adequately investigated for any of the vitiviruses members so far.

The aim of the research described in this paper was to gain further insight in distribution profile of the ORF3 encoded protein in infected cells. To the best of our knowledge, this is the first work describing experimentally derived properties that show how the protein encoded by ORF3 of vitiviruses is involved in virus movement. We addressed the targeting of the MP of GVA, as well as the MP of the GVB, to the cell periphery of *Nicotiana benthamiana* and grapevine protoplasts. As a tool we used fluorescent-tagged MPs expressed in cells through GVA chimeric vectors. We found that the fluorescent-tagged MP was located in fluorescent peripheral punctate and tubule-like structures and localized to the plasmodesmata. The effect of several metabolic inhibitors, which depolymerize the cytoskeleton, on the formation of punctate and tubule-like structures, was studied.

2. Materials and methods

2.1. Construction of plasmids

Schematic illustrations of constructs used in this study are presented in Fig. 1. Plasmids were constructed using standard cloning methods (Sambrook et al., 1989) and transformed using *Escherichia coli* strains JM109 or DH5α or the *Agrobacterium tumefaciens* strain EHA 105. The binary vector pCAMP-35S (Moskovitz et al., 2008), was used for the transient expression experiments. PCR amplifications were performed with the use of the *PfuTurbo* DNA polymerase (Stratagene) or the Deep-Vent DNA polymerase (New England Biolabs, Inc.). The sequences of the red fluorescent protein (RFP), cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP) were amplified by PCR utilizing the constructs described by Chung et al. (2005) and Tzfira et al. (2005), as templates.

The clones pGR5 and pGVA118, which represent a GVA full-length infectious clone and a GVA-based expression vector, respectively (Galiakparov et al., 2003a; Haviv et al., 2006), were used as templates for PCR amplifications and for expression of non-viral gene products. To prepare the construct GVA-MP-GFP-177, the enhanced green fluorescent protein (GFP) gene (Yang et al., 1996) was fused in frame to the 3′-end of the ORF3 in pGR5. Then a GVA fragment of 180 nts, which is predicted to contain the CP-sgRNA promoter, was inserted upstream of the CP gene (ORF4). The construct GVA-GFP-164 was prepared by insertion of the enhanced GFP gene in the GVA118 expression vector, between the *Not*I and *Apa*I digestion sites. The construct GVA-MP-GFP-229 was made by deleting the amino acids (aa) 29–116 of the MP of the GVA-MP-GFP-177. The construct GVA-MP-RFP-231 was prepared in a similar manner to the construct GVA-MP-GFP-177, but with the use of the RFP-encoding gene. The sequences encoding the fusion proteins MBD-GFP and YFP-Talin (Pouwels et al., 2002) were inserted in the GVA118 expression vector between the *Not*I and *Apa*I digestion sites, to generate the constructs GVA-GFP-MBD-373 and GVA-YFP-TALIN-374, respectively. The construct GVA-GFP-469 is similar to GVA-GFP-164, but assembled under control of the CaMV 35S promoter into the binary vector pCAMP-35S (Moskovitz et al., 2008). The construct GVA-GFP-ΔCP-524 was prepared by disrupting the CP frame in the construct GVA-GFP-469 by *Eco*NI digestion followed by filling with the klenow enzyme and re-ligation. In the resultant clone, the translation of the CP would be truncated nearly in the middle of the gene to produce 101 aa residues out of 198 aa of the complete protein. The construct GVB-MP-GFP-83 was prepared on the basis of our GVB infectious clone (Moskovitz et al., 2008) by deleting the CP gene and insertion of the GFP gene in fusion to the 3′-end of the MP gene. The construct GVB-MP-GFP-86, is similar to the previous construct but assembled under the CaMV 35S promoter into the binary vector pCAMP-35S (Moskovitz et al., 2008). The constructs MP-RFP-387, MP-CFP-491, CP-YFP-471 and TMV-MP-YFP-387 were generated by cloning of the fusion proteins GVA MP-RFP, GVA MP-CFP, GVA CP-YFP and TMV-MP-YFP, respectively in the binary vector pCAMP-35S (Moskovitz et al., 2008).

2.2. Plant growth conditions

N. benthamiana plants were grown in commercial potting soil at 26 °C. Plants, 5–6 weeks old, were used for viral RNA inoculation and agroinfiltration. Plants, 8–9 weeks old, were used for the preparation of protoplasts.

2.3. In vitro transcription, inoculation of protoplasts, and treatment with inhibitors

The constructs assembled under the control of the T7 RNA promoter (Fig. 1) were used for *in vitro* RNA transcription as described in Haviv et al. (2006). *N. benthamiana* mesophyl protoplasts were isolated and transfected by polyethylene glycol-mediated transformation as described (Haviv et al., 2006). In the inhibitor assays, the protoplasts were resuspended after transfection into 1 ml protoplast culture medium and divided onto three aliquots. One of the aliquots was left untreated. To the other two aliquots, Oryzalin (50 μM or 100 μM) and Cytochalasin D (500 μM or 1 mM) were added immediately after the transfection process. The protoplasts were incubated for 72 h under fluorescent light at 26 °C.

Grapevine mesophyl protoplasts were isolated from leaves of virus-free *Vitis vinifera* cv. Prime as described previously (Theodoropoulos and Roubelakis-Angelakis, 1990). Transfection of protoplasts was carried out by polyethylene glycol-mediated transformation, similarly to *N. benthamiana* protoplast transfection.