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On the interaction and localization of the beet necrotic yellow vein virus replicase

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ABSTRACT

Beet necrotic yellow vein virus (BNYVV) is a multipartite positive-strand RNA virus. BNYVV RNA-1 encodes a non-structural p237 polyprotein processed in two proteins (p150 and p66) by a *cis*-acting protease activity. BNYVV non-structural proteins are closely related to replication proteins of positive strand RNA viruses such as hepeviruses rather to other plant virus replicases. The p237 and dsRNA have been localized by TEM in ER structures of infected leaf cells whereas dsRNA was immunolabeled in infected protoplasts. The p150 contains domains with methyltransferase, protease, helicase and two domains of unknown function whereas p66 encompasses the RNA-dependent RNA-polymerase signature. We report the existing interactions between functional domains of the p150 and p66 proteins and the addressing of the benyvirus replicase to the endoplasmic reticulum. Yeast two-hybrid approach, colocalization with FRET-FLIM analyses and co-immunoprecipitation highlighted existing interactions that suggest the presence of a multimeric complex at the vicinity of the cellular membranous web.

1. Introduction

Positive-strand RNA viruses represent the most abundant viruses infecting plants (Schwartz et al., 2002). For their multiplication, these viruses require the immediate expression of a dedicated RNA-dependent RNA-polymerase (RdRp) in order to specifically ensure their genome recognition and amplification. For this purpose, RdRp interacts with other early expressed viral proteins and participates to the assembly of the replication complex. Such complex contains helicase and ATPase activities and when concerned, a methyltransferase functions to allow capping of genomic RNAs for cytoplasmic replicating viruses having a 5′ cap structure.

Up to now, replication complexes have always been associated to single- or double-membrane vesicles or invaginations issued from virus-induced rearranged membranous cell structures (den Boon et al., 2010; Romero-Brey and Bartenschlager, 2014). Such membrane associations are thought to enhance the replication efficiency and to protect the viral genome and anti-genome RNAs from innate cellular responses. However, membrane association of the replication complex varies and depends on the virus genus. Flock house virus (FHV) replication complex is associated with mitochondrial outer membrane invagination (Miller and Ahlquist, 2002; Miller et al., 2001), turnip yellow mosaic virus (TYMV) with chloroplast membrane invaginations (Prod'homme et al., 2001; Singh and Dreher, 1997) while *Picornaviridae* member species often target the endoplasmic reticulum (ER) membrane (Bienz et al., 1983, 1992; Echeverri and Dasgupta, 1995; Teterina et al., 1997) like brome mosaic virus (BMV) replication complex that induces ER invaginations (Schwartz et al., 2002) or tobamovirus replication takes place as well on host membranes (Ishibashi et al., 2012) while hijacking TOM1 and TOM2A host proteins (Nishikiori et al., 2006; Verchot, 2011).

Expression strategies of replication-involved proteins vary from the production of polypeptides from distinct open reading frames (ORF) physically separated on distinct RNA species (*e.g.* BMV 1a and 2a proteins (Ahlquist et al., 1987)), and can be separated one another either by a read-through stop codon as for tobacco mosaic virus (TMV) (Beier et al., 1984), by a frameshift region as retrieved in the genome of *Luteoviridae* members (Prufer et al., 1992) or

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expressed as a polyprotein further cleaved into functional proteins (*e.g.* TYMV (Jakubiec et al., 2004, 2007; Rozanov et al., 1995)). Thus, replication complex formation requires the correct addressing of the participating proteins to the target subcellular membrane that also involve the recruitment of viral and cellular factors. TYMV replication complex formation requires the interaction of the proteolytic cleavages product of the polyprotein together with the addressing of the complex to the chloroplast outer membrane, which induces invaginations (Jakubiec et al., 2004). Similarly, BMV complex formation requires the interaction between 1a and 2a proteins at the ER membrane and produces spherules (Kao and Ahlquist, 1992; Kao et al., 1992; Restrepo-Hartwig and Ahlquist, 1996). Hence, successful characterization of protein interactions and membrane association of replication complexes have emerged in the literature (den Boon et al., 2010; Mine and Okuno, 2012). However, little is known about the replication complex formation and localization of the new *Benyviridae* family members and of the *Benyvirus* genus representative member, Beet necrotic yellow vein virus (BNYVV) (Gilmer and Ratti, 2012).

BNYVV is a multipartite positive strand RNA virus, the genome of which is split into four to five RNA species. All genomic RNAs possess a 5′ Cap and a polyA tail that make the viral genome resemble cellular mRNAs. BNYVV RNA-1 is responsible for the replication complex expression as this species is necessary and sufficient for viral RNA-1 amplification in protoplasts (Gilmer et al., 1992). RNA-1 encodes a single polyprotein of 237 kDa (p237) further processed by protease activity into 150 and 66 kDa proteins (Hehn et al., 1997). Phylogenetic analyses highlighted the closer relationship between BNYVV p237 with *Hepeviridae* and *Togaviridae* family replicase proteins than with other plant virus replicases (Kondo et al., 2013; Koonin et al., 1992).

In this paper, we have localized the replicase protein and dsRNA in infected cells using immunolabeling. Individually expressed p237 protein domains were used to identify p237 regions that could be involved in protein–protein interaction allowing a replication complex formation. The subcellular localizations of the polyprotein domains expressed as fluorescent fusion proteins in and out of the viral context have been studied. The interactions identified using yeast two hybrid were confirmed by subcellular localization, followed by Fluorescence Resonance Energy Transfer–Fluorescence-Life time Imaging Microscopy (FRET-FLIM) analyses and co-immunoprecipitation approaches.

2. Materials and methods

2.1. Cloning procedures

The descriptive analysis of the *Hepatitis E virus* replicase protein (Ahmad et al., 2011) was used to design primers (Supplementary Table 1) allowing an in-frame fusion of nine p237 sequence domains to the 3′ region of the GAL4-BD in pGBK-T7 vector using *Eco*RI and *Sal*I sites (Clontech). *Eco*RI-*Sal*I fragments issued from pGBK-T7 constructs were further subcloned in frame with GAL-4AD in pGAD-T7 using *Eco*RI and *Xho*I (Clontech). GAL4-BD and GAL4-AD proteins contain a Myc- and HA-tag respectively provided by the vectors. Fragments containing either an HA or Myc tag were PCR amplified using *Sun*I and *Xba*I restriction site-containing oligonucleotides (Supplementary Table 1) and introduced into *Bsr*GI-*Xba*I digested pRep5EGFP. Sub-cloning of fragments into pRep3mRFP was performed using *Xma*I and *Bam*HI restriction sites (Supplementary Table 1) (Schmidlin et al., 2005). The sequences of interest were digested with *Spe*I and *Bam*HI restriction enzymes and introduced into *Xba*I-*Bam*HI digested pBin61 (Voinnet et al., 1998). The nucleotide sequences of the entire set of clones were verified.

2.2. Yeast two hybrid

The interaction studies were initially performed using a mating approach of AH109 yeast carrying pGBK-T7 constructs with Y187 yeast carrying pGAD-T7 fusion proteins as described previously (Klein et al., 2007). To validate our observations in a homozygote genetic background, interactions were further verified by co-transformation of AH109 strain with pGBK-T7 and pGAD-T7 combinations using Clontech Matchmaker protocol. Empty vectors were used as negative controls. Positive control was obtained with pVA3 (Iwabuchi et al., 1993) and pTD1(Li and Fields, 1993) plasmids expressing the murine p53 and the SV40 largeT antigen, respectively. The strength of the interaction were evaluated by the growth of DO_{600} 0.3, 0.03 and 0.003 yeast dilution drops of 3 μl on selective media lacking tryptophane, leucine and histidine (SD-WLH) containing increasing amounts of 3-amino-triazol (3-AT) or depleted with Adenine (SD-WLAH).

2.3. Agroinfiltration, CLSM and FRET-FLIM analyses

Leaves of 5-leaf stage *Nicotiana benthamiana* plants were co-infiltrated with combinations of *Agrobacterium tumefaciens* strains carrying binary vectors (Voinnet et al., 1998). Agrobacteria carrying pBin-EGFP-, pBin-mRFP-fused sequences or pBin mCherry-HDEL (Nelson et al., 2007) were infiltrated together with a binary vector allowing the expression of the Tombusvirus p19 viral suppressor of RNA silencing (VSR) to insure the production of the fusion proteins (Voinnet et al., 2003). When specified, agrobacteria carrying a binary vector expressing full-length cDNA copy of BNYVV RNA-1 was added to provide a viral context in the infiltrated tissues (Delbianco et al., 2013). The localization of fusion proteins was determined as described (Erhardt et al., 2005) using a ZeissLSM700 confocal microscope two days post-infiltration (p.i.). FLIM studies were performed as described previously (Chiba et al., 2013). FRET-FLIM analyses were performed two days p.i.

2.4. Co-immunoprecipitation

Total proteins were extracted from 0.3 g of agroinfiltrated leaves two days post-infiltration (dpi) using Miltenyi μMACS epitope tag protein isolation kit (Miltenyi Biotec SAS, France) according to the manufacturer's instructions. Briefly, anti-EGFP microbeads were incubated with 1.1 mL of total leaf protein extract (Tris–HCl pH 8.0, 50 mM; NaCl 150 mM, Triton X-100 1%, containing protease inhibitor cocktail) for 30 min at 4 °C and captured using a magnetic field. After washes (Tris–HCl pH7.5, 20 mM), the co-IP fractions were eluted in 100 μL according to the manufacturer's instructions. Input, unbound and elution protein contents were separated by SDS-PAGE and analyzed by western blotting with an mRFP polyclonal antibody.

2.5. Plant, protoplast infection and analyses procedures

Chenopodium quinoa protoplasts preparation and infection were performed as previously described (Veidt et al., 1992). Infection procedures using *in vitro* runoff transcripts, northern and western blot analyses were performed as described previously (Klein et al., 2007).

2.6. Immunolocalization procedures

C. quinoa protoplasts were immobilized on poly-L-lysine hydrobromide coated glass slides and fixed for 60 min in 4% paraformaldehyde diluted in the protoplast incubation medium. Unspecific binding was blocked by incubation in PBS 1× containing 2.5% bovine serum albumin, 5% skimmed milk powder and