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Live cell imaging of interactions between replicase and capsid protein of Brome mosaic virus using Bimolecular Fluorescence Complementation: Implications for replication and genome packaging

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ABSTRACT

In *Brome mosaic virus*, it was hypothesized that a physical interaction between viral replicase and capsid protein (CP) is obligatory to confer genome packaging specificity. Here we tested this hypothesis by employing Bimolecular Fluorescent Complementation (BiFC) as a tool for evaluating protein–protein interactions in living cells. The efficacy of BiFC was validated by a known interaction between replicase protein 1a (p1a) and protein 2a (p2a) at the endoplasmic reticulum (ER) site of viral replication. Additionally, co-expression *in planta* of a bona fide pair of interacting protein partners of p1a and p2a had resulted in the assembly of a functional replicase. Subsequent BiFC assays in conjunction with mCherry labeled ER as a fluorescent cellular marker revealed that CP physically interacts with p2a, but not p1a, and this CP:p2a interaction occurs at the cytoplasmic phase of the ER. The significance of the CP:p2a interaction in BMV replication and genome packaging is discussed.

Introduction

The biological function of a given protein is determined by the formation of stable or transient protein complexes and networks. Consequently, disruption of protein complex formation or network leads to abnormal development of the host or may lead to disease induction. Thus, evaluation and identification of protein–protein interactions (PPI) often provide novel insight into their regulatory function in several signaling processes. Techniques such Yeast Two-Hybrid (YTH), Fluorescence Resonance Energy Transfer (FRET) and co-immuno precipitation (Co-IP) are frequently used for evaluating PPI (Khan et al., 2011). YTH analyses have provided invaluable information about interacting proteins in stable or transient complex formation, but an inherent disadvantage of YTH is that a large number of interactions are predicted to be false positives. Although FRET is ideal for visualizing PPI in real time, determination of protein interactions by FRET requires ratiometric image analysis to subtract background signals. Despite their usefulness, YTH and FRET do not monitor the dynamics of interaction and localization *in vivo* in real time. This information is necessary in order to understand protein function at the cellular, tissue and organism levels. In recent years, the Bimolecular Fluorescent Complementation assay (Citovsky et al., 2006; Kerppola, 2008) has gained momentum in evaluating PPI *in vivo*. When combined with fluorescently labeled cellular marker proteins, BiFC offers the advantage of precisely determining the subcellular localization of PPI. Availability of vectors amenable for engineering fusion proteins followed by their expression *in planta* (Citovsky et al., 2006) is particularly attractive for testing PPI in plant viruses.

Brome mosaic virus (BMV) is the type of species of the genus *Bromovirus* (King et al., 2011), and belongs to the *Bromoviridae* family of plant viruses. The genome of BMV is divided among three RNA components. Viral replication is dependent on two non-structural proteins, p1a (containing an RNA-helicase-like domain and a capping domain) and p2a (containing a polymerase domain) encoded respectively by genomic RNAs 1 and 2 (Ahlquist, 2006). Genomic RNA3 is dicistronic, encoding a non-structural movement protein (MP) and the capsid protein (CP) which is expressed *via* a subgenomic RNA (RNA4) produced during replication (Ahlquist, 2006). Replication of BMV has been studied in detail at the molecular and subcellular level using natural plant hosts (Bamunusinghe et al., 2011; Kao and Sivakumaran, 2000) and non-host, surrogate yeast system (Ahlquist, 2006).

Macromolecular interactions (*e.g.* PPI, protein–RNA interactions) have been shown to be intimately involved in the establishment of a successful infection by an RNA viral pathogen (Hunter, 1994; Kujala et al., 2001). Although virus-encoded proteins are

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envisioned to perform a specific function (*e.g.* viral replicase in catalyzing the synthesis of progeny RNA), accumulated information over the past two decades revealed otherwise (Laliberte and Sanfacon, 2010). For example, in addition to synthesizing viral progeny RNA, viral replicases have been shown to be intimately associated with many important functions such as RNA silencing (Ding et al., 2004), symptom modulation and movement (Creager et al., 1999), genome packaging and translation (Sanz et al., 2007). Another important multifunctional macromolecular entity is the CP. The primary function of the CP is to encapsidate the infectious genome progeny and form stable virions (Rao, 2006). Several factors such as CP–CP interactions, sequence-independent RNA–protein interactions (involved in stabilization of encapsidated virions), sequence-dependent RNA–protein interactions (origin of assembly sequences), auxiliary factors such as cellular tRNAs, viral replicase and scaffolding protein contribute to the assembly of infectious virions (Rao, 2006). Experimental evidence suggested that packaging specificity in BMV and *Flock house virus* (FHV) is regulated not only by synchronized co-expression of homologous replicase and CP, but also the translation of CP from replication derived mRNA (Rao, 2006). In addition, we for BMV (Bamunusinghe et al., 2011) and others for FHV (Venter et al., 2009) showed that the subcellular localization sites of CP and replication overlap. Subsequent follow up studies further revealed that, in FHV, a physical interaction between replicase and CP is obligatory to confer packaging specificity (Seo et al., 2012). However, in BMV, unlike FHV, functional replicase is a complex of two non-structural proteins, p1a and p2a (Kao and Sivakumaran, 2000). If packaging specificity in BMV, like in FHV, requires a replicase–CP interaction, the question that needs to be addressed would be: *which of the two proteins interact with CP?* Thus, to find an answer to this question, in the present investigation, we opted to employ BiFC in conjunction with endoplasmic reticulum (ER) labeled with mCherry as a cellular marker protein. Our results

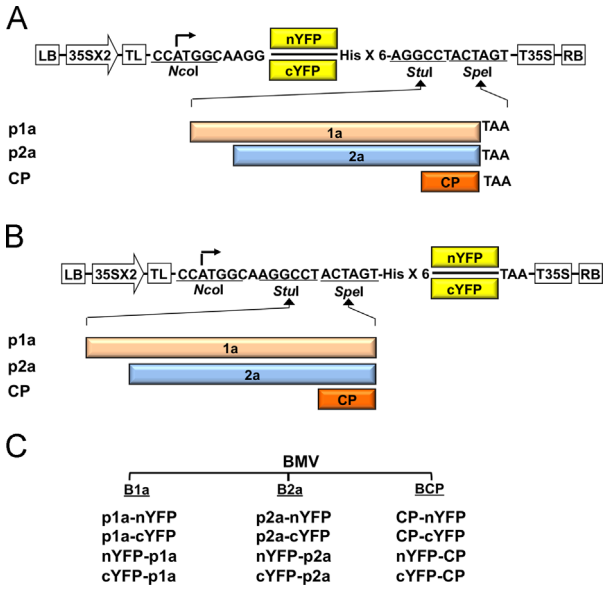

Fig. 2. Schematic representation of fluorescent protein fusion constructs used in the present study. Two pairs of basal BiFC binary vectors were used. Constructs shown in panels A and B were engineered respectively for generating N-terminal (PZPn-nYFP and PZPn-cYFP) and C-terminal fusions (PZPc-nYFP and PZPc-cYFP). Open reading frames (ORFs) of BMV p1a, p2a and CP were fused in-frame to each pair of binary vectors using *Stu*I and *Spe*I sites. Each binary vector contained in sequential order, a left border of T-DNA (LB); a double 35S promoter (35Sx2); a tobacco etch virus (TEV) translation enhancer leader sequence (TL), multiple cloning site, a fragment of N-terminal 157 residues of yellow fluorescent protein (nYFP), a fragment of C-terminal 83 residues of YFP (nYFP), six-histidine tag (Hisx6), a 35S terminator (T35S), and a right border of T-DNA (RB). (C) Four possible fusion constructs for p1a, p2a and CP tested in this study are shown (Fusion Proteins).

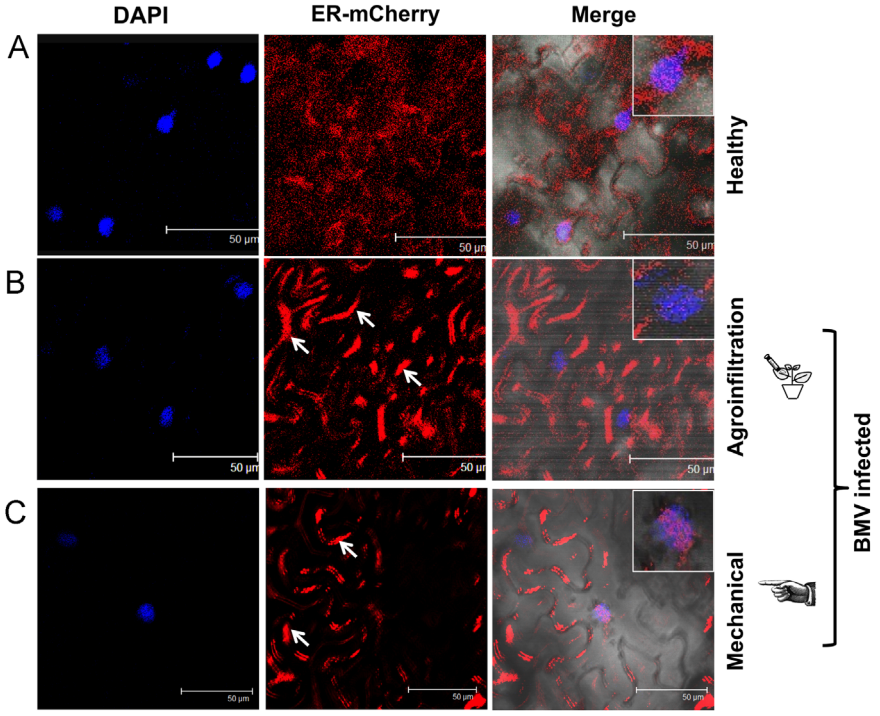

Fig. 1. Visualizing ER rearrangement by confocal microscopy using mCherry labeled ER marker protein. *N. benthamiana* plants were agroinfiltrated (A) with a binary construct of ER-mCherry or (B) with a mixture containing binary constructs of all three wild type BMV RNA and ER-mCherry or (C) with ER-mCherry at 1 day post-mechanical inoculation with purified BMV virions. At 4 dpi, infiltrated leaves were stained with DAPI as a nuclear marker and observed under a confocal microscope equipped with a specific laser/filter combination to detect blue fluorescence emitted by DAPI (excitation at 345 nm) and red fluorescence emitted by mCherry (excitation at 587 nm). Insets (A–C): a magnified view of ER localization in the peri-nuclear area, Bar, 50 µm. In panels B and C, arrows indicate rearranged ER.