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The VP37 protein of *Broad bean wilt virus 2* induces tubule-like structures in both plant and insect cells

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ABSTRACT

VP37 protein of *Broad bean wilt virus 2* (BBWV-2) is a multifunctional protein that binds single-strand nucleic acids, interacts with viral coat protein (CP) and potentiates the virus cell-to-cell movement in its host plant. In this study, tubule-like structures filled with virus-like particles were observed by Electron Microscopy in plasmodesmata in walls of *Chenopodium quinoa* leaf cells infected with BBWV-2. Immunogold labeling using VP37 protein specific antibody demonstrates that the VP37 is a component of the tubular structures. When VP37 was fused with the green fluorescent protein (VP37-GFP) and expressed in BY-2 protoplasts or in insect Tn cells, green fluorescent tubules of various lengths were produced, protruding from the surface of the expressing cells. These findings suggest that the movement of BBWV-2 between cells is mediated by the tubular structures that contain the VP37 protein, and the VP37 protein itself is capable of inducing these tubule-like structures in cells. Our results also suggest that the plant and insect cell factors involved in the tubule formation have conserved features.

1. Introduction

Virus cell-to-cell movement is considered to be an active process that is mediated by one or more viral proteins, known as movement proteins (MPs) (Boevink and Oparka, 2005; Scholthof, 2005; Hofmann et al., 2007). MPs themselves, or together with some other viral protein(s), can modify the plasmodesma (PD) in walls of plant cells to assist virus transport from the infected cells to neighboring uninfected cells (Carrington et al., 1996; Nelson and Citovsky, 2005). To date, two basic strategies for MPs have been identified (Lucas, 2006; Melcher, 2000; Scholthof, 2005). The first strategy is exemplified by the *Tobacco mosaic virus* (TMV) MP, which increases the size exclusion limit (SEL) of the PDs without major changes of its structure (Boyko et al., 2000; Deom et al., 1987; Kawakami et al., 2004). The second strategy for MPs, exemplified by the MP of *Cowpea mosaic virus* (CPMV), induces tubule-like structures that traverse the PDs in cell walls and mediate the virus cell-to-cell movement (Bertens et al., 2000; Gopinath et al., 2003; Kasteel et al., 1997). Several other plant viruses including *Grapevine fan leaf virus* (GFLV), *Cauliflower mosaic virus* (CaMV) and *Apple latent spherical virus* (ALSV) were also shown to produce virus particle-containing tubule-like structures. The presence of virus particles in these tubules suggests strongly that these viruses traffic between cells as virions (van Lent et al., 1991; Ritzenthaler et al., 1995; Yoshikawa et al., 2006). Using immunogold labeling and Electron Microscopy or transient expression of GFP tagged MPs of these viruses, it was determined that the MPs of CPMV, *Tomato spotted wilt virus* (TSWV), CaMV and *Apple chlorotic leaf spot virus* (ACLSV) were the major components of the tubules (Huang et al., 2000; Kasteel et al., 1996; Satoh et al., 2000; Storms et al., 1995).

Little is known about the cell-to-cell movement of *Broad bean wilt virus 2* (BBWV-2). BBWV-2 is an RNA virus belonging to the genus *Fabavirus* in the family *Comoviridae* (Fauquet et al., 2005). The virus consists of two positive sense single stranded RNAs of 6.0 and 3.6 kb long packaged in 25 nm icosahedral virions (Zhou and Li, 1996). The VP37 protein encoded by the 3.6 kb RNA2 was previously identified as the MP of BBWV-2. The VP37 protein was shown to bind single-strand nucleic acids *in vitro* (Qi et al., 2002) and interacts specifically with the small coat protein of BBWV-2 (Liu et al., 2009a). The GFP tagged VP37 protein was shown to localize at the cell periphery in agroinfiltrated *Nicotiana benthamiana* leaf cells (Liu et al., 2009a). However, how VP37 functions during BBWV-2 cell-to-cell movement is not clear. It was suggested that the VP37 protein could form tubule-like structures similar to that produced by other viruses such as CPMV and GFLV in the *Comoviridae*. Here we report that tubule-like structures containing BBWV-2 virions were observed in PDs across the cell walls in BBWV-2 infected *Chenopodium quinoa* leaves through Electron Microscopy. When

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the GFP tagged VP37 protein (VP37-GFP) was transiently expressed in either BY-2 protoplasts or *Trichoplusia ni* (Tn-5B1-4, Tn) cells, the green fluorescent tubule-like structures were observed protruding from the surface of both transfected cells.

2. Materials and methods

2.1. Virus, host plant, and antibody

BBWV-2 isolate PV131 used in this study was obtained from Prof. Vittoria Lisa (Istitio Di Fitovirologia Applicata, Italy), and was maintained in *C. quinoa*. Two-week-old *C. quinoa* or *Vicia faba* seedlings were mechanically inoculated with BBWV-2 and the inoculated plants were grown in a greenhouse set at 26 °C. Polyclonal antibody against the VP37 protein was prepared previously in the lab as described (Qi et al., 2002).

2.2. Tissue embedding, sectioning and transmission electron microscopy

For ultrastructural analysis, tissue samples were cut from the systemic leaves of BBWV-2 infected *C. quinoa* plants at 10 days post inoculation (dpi). The sampled tissues were fixed in 2.5% glutaraldehyde and 1% osmium tetroxide [both in 100 mM phosphate buffer (PB), pH 7.0] as described (Liu et al., 2009b). The fixed tissues were then embedded in Epon 812 resin as instructed by the manufacture (SPI-EM, Division of Structure Probe, Inc., West Chester, USA). Untrathin sections (70 nm) were cut from the embedded tissues using the Ultrcut E ultramicrotome (Reichart-Jung, Vienna, Austria) and mounted on formvar-coated grids.

For immunogold labeling, the collected tissue samples were fixed in PB (pH 7.2) containing 1% glutaraledhyde and 4% paraformaldehyde for 3 h at 4 °C after a brief vacuum infiltration. The fixed tissues were washed three times in the PB and then dehydrated through a series of graded ethanol solutions at −20 °C. The tissues were transferred into LR White resin (The Londong Resin Co. Ltd., London, UK) and polymerized under the ultraviolet light (360 nm wavelength) at −20 °C as described (Castellano et al., 2005). Untrathin sections were cut and mounted on formvar-coated nickel grids. The sections were probed with the anti-VP37 antibody followed by the 10 nm protein A-gold conjugate (Sigma, St. Louis, MO) as described previously (Xiong et al., 2009). Sections probed with the bovine serum albumin (BSA) followed by the protein A-gold conjugate was used as controls.

After double staining with lead citrate and uranyl acetate, both Epon 812 and LR White embedded ultrathin sections were examined under a JEM-1230 TEM (JEOL, Akishma, Japan). Images of the sections were captured using a Gatan 792 CCD camera (Gatan, CA, USA).

2.3. Transient expression of VP37-GFP in BY-2 protoplasts

The plasmid $pCHF_3$-VP37-GFP (Liu et al., 2009a) was transiently expressed in BY-2 protoplasts by electroporation as described (Miao and Jiang, 2007). Briefly, 30 mL 4-day-old suspension cells were centrifuged at 800 rpm for 2 min and the pellet was resuspended in 10 mL enzyme solution. 400 μL pre-cold protoplasts (1×10^6 mL^{-1}) were mixed with 20 μg plasmid DNA and used for electroporation. The transfected protoplasts were resuspended in 1.5 mL protoplast culture media and added to a petri dish and cultured in the dark at 26 °C.

2.4. Expression of VP37-GFP fusion protein in insect cells

The VP37-GFP fusion protein was expressed in *T. ni* (Tn) cells using a Bac-to-Bac baculovirus expression system as described (Invitrogen, Carlsbad, CA, USA). The VP37-GFP fragment was PCR amplified from the $pCHF_3$-VP37-GFP using primers VP37-XbaI-F (5′-GTCTAGAATGAATGAGGCAAATATC-3′) and GFP-XhoI-R (5′-CGCTCGAGTTACTTGTACAGCTCGTCC-3′). The PCR product was digested with the XbaI and XhoI restriction enzymes and inserted into the XbaI/XhoI site within pFastBac™ 1 shuttle vector. The recombinant Bacmid, referred to as BavVG, was isolated and used to transfect Tn cells using lipofectin as instructed (Invitrogen). The Tn cells were infected with the recombinant baculouvirus at a multiplicity of infection of 10 and then incubated at 27 °C for 3 days. The culture supernatants were subsequently harvested for Confocal Microscopy or stored at 4 °C for future infections.

2.5. Confocal laser scanning microscopy

The transfected BY2 protoplasts and Tn cells were examined for VP37-GFP expressing using a confocal laser scanning microscope (CLSM, Leica TCS SP5, Mannheim, Germany). The excitation and emission wavelengths were set at 488 and 500–550 nm, respectively. All TEM and CLSM images were further processed using the Adobe Photoshop version 6.0 software (Adobe Systems Inc., San Jose, CA, USA).

3. Results

3.1. Tubule-like structures in cells of BBWV-2 infected leaf tissues

Both *C. quinoa* and *V. faba* were inoculated with BBWV-2. Severe systemic necrosis appeared on *V. faba* plants by 5 dpi and most of these infected plants wilted within 2 weeks post infection. Therefore *C. quinoa*, also a systemic host of BBWV-2, was chosen as the host plant for our Electron Microscopy. In virus inoculated *C. quinoa* leaves, chlorotic lesions were first seen by 3 dpi. Chlorotic spots in systemically infected *C. quinoa* leaves appeared by 10 dpi. When thin sections from the Epon 812 embedded tissues were examined using an electron microscope, the cells in BBWV-2 infected leaves showed areas with proliferated membranes. These membrane-proliferated areas contained large vesicles and electron-dense materials. In these infected cells, large virus aggregates similar to the size of chloroplasts were also observed (Fig. 1A). In the cytoplasm of the infected cells, numerous tubular structures of 75–80 nm in diameter were seen and each transverse section of the tubules contained approximately 9 virus-like particles (Fig. 1B and C). This type of tubular structure was previously thought as a form of virus accumulation and had no relationship with virus movement (Hong et al., 2006; Liu et al., 2009b). A second type of tubular structures was also observed in these infected cells. These tubules also contained virus-like particles and appear to be protruding from or penetrating into the PD in walls between two cells. Most of the tubules observed appeared to traverse the entire PDs (Fig. 1D–F, arrows). Virus-like particles in these tubules were approximately of 25 nm in diameter and arranged in single lines in the tubules. Diameter of the PDs containing tubules ranged from 50 to 60 nm and is significantly larger than that for the PDs without the tubules (20–30 nm) (Fig. 1G). These observations suggest that BBWV-2 moves between cells as virions through the tubules.

To confirm that the VP37 protein was present within the PDs that contained the tubules, sections from the LR White resin embedded tissues were probed with the anti-VP37 polyclonal antibody followed by the 10 nm immunogold conjugate. The immunogold particles were seen predominantly at the cell periphery and in the PDs using the electron microscope (Fig. 1H and I). No gold particles were observed in PDs in sections from the uninfected leaf tissues (Fig. 1J). Our results indicate strongly that the VP37 protein is a component of the tubules.