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Cell-to-cell trafficking, subcellular distribution, and binding to coat protein of *Broad bean wilt virus 2* VP37 protein

Chengke Liu[a], Chunmei Meng[a], Li Xie[a], Jian Hong[a,b,*], Xueping Zhou[a,*]

[a] State Key Laboratory of Rice Biology, Institute of Biotechnology, Zhejiang University, Hangzhou 310029, People's Republic of China
[b] Center of Analysis and Measurement, Zhejiang University, Hangzhou 310029, People's Republic of China

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ABSTRACT

Broad bean wilt virus 2 (BBWV 2) is a member of the genus *Fabavirus* of the family *Comoviridae*. To date, a movement protein (MP) of BBWV 2 has not been described. Here we demonstrate that the green fluorescent protein (GFP)-VP37 fusion protein can move from initial bombarded cells to neighboring cells in *Nicotiana benthamiana* epidermal leaves. In addition, the GFP-VP37 fusion protein localizes as a halo around the nucleus and as punctate spots on the cell periphery in *N. benthamiana* epidermal leaf cells and BY-2 suspension cells. Fluorescence near the nucleus also was co-localized with the endoplasmic reticulum in BY-2 cells. Fibrous networks were found in GFP-VP37 agro-infiltrated *N. benthamiana* epidermal leaf cells. Deletion analyses indicated that the C-terminal region of the VP37 protein is essential for localization at the cell periphery. Using a blot overlay assay and bimolecular fluorescence complementation assay, the purified 6×His-tagged VP37 protein was shown to bind specifically to the small coat protein of BBWV 2. The above results indicate that VP37 is a movement protein.

1. Introduction

Plant virus movement within host plants involves in two distinct steps, cell-to-cell movement and long-distance systemic transport (Carrington et al., 1996; Boevink and Oparka, 2005). Cell-to-cell movement is considered to be an active process that is mediated by one or more viral encoded proteins, called movement proteins (MPs) (Lazarowitz and Beachy, 1999; Boevink and Oparka, 2005; Scholthof, 2005; Hofmann et al., 2007). Plant virus MPs are reported to be multifunctional. For example, the tobacco mosaic virus (TMV) 30K MP anchors to plasmodesma (PD) and modifies PD to enable transport of viral RNA/protein complexes from infected to neighboring uninfected cells (Melcher, 2000; Boevink and Oparka, 2005). Viruses encoding more than one MP have these functions split among two or three proteins (Koh et al., 2001; Morozov and Solovyev, 2003; Scholthof, 2005; Verchot-Lubicz, 2005). The MPs can bind single-strand nucleic acids, also mediate self transport from cell to cell (Schoumacher et al., 1992; Wieczorek and Sanfacon, 1993; Melcher, 2000; Satoh et al., 2000; Cowan et al., 2002; Carvalho et al., 2004; Navarro et al., 2006), some MPs or complexes can bind specifically to heterologous coat protein (CP) to form complexes that function in movement (Carvalho et al., 2003; Sánchez-Navarro et al., 2006).

Broad bean wilt virus 2 (BBWV 2), a member of the genus *Fabavirus* of the family *Comoviridae* (Fauquet et al., 2005), possesses a bipartite, single-stranded, positive sense RNA genome (Qi et al., 2000a,b,c). Both RNA1 and RNA2 encode polyproteins and processing of the RNA1-encoded polyprotein yields a set of proteins required for viral replication while the cleavage of the RNA2-encoded polyprotein produces the 44 kDa large coat protein (LCP), 22 kDa small CP (SCP) and 53 kDa/37 kDa proteins (designated as VP53/37) (Fig. 1A). The VP37 protein, a stable final maturation product of RNA 2 (Qi et al., 2000b), is supposed to be a MP based on the following evidence: (a) The genome of BBWV 2 shares the same translation strategy as cowpea mosaic virus (CPMV) of the genus *Comovirus* of the family *Comoviridae*, and the position of VP37 protein in the polyprotein encoded by RNA 2 is the same as the 48 kDa MP of CPMV (Qi et al., 2000a,b; Pouwels et al., 2003). (b) The VP37 protein can bind single-strand nucleic acids in vitro (Qi et al., 2002). (c) The VP37 protein contains motifs conserved in MPs of some plant viruses (Koh et al., 2001; Zhou et al., 2001). Among three genera in the *Comoviridae*, the MPs of comoviruses and nepoviruses have been studied in detail (Pouwels et al., 2002, 2004; Gopinath et al., 2003; Laporte et al., 2003), however, little is known about MP of fabaviruses. In this study, the green fluorescent protein (GFP) was used as a report gene in order to observe cell-to-cell trafficking and subcellular localization of the BBWV 2 VP37 protein. We found that

* Corresponding authors. Institute of Biotechnology, Zhejiang University, Hangzhou 310029, People's Republic of China.
E-mail addresses: jhong@zju.edu.cn (J. Hong), zzhou@zju.edu.cn (X. Zhou).

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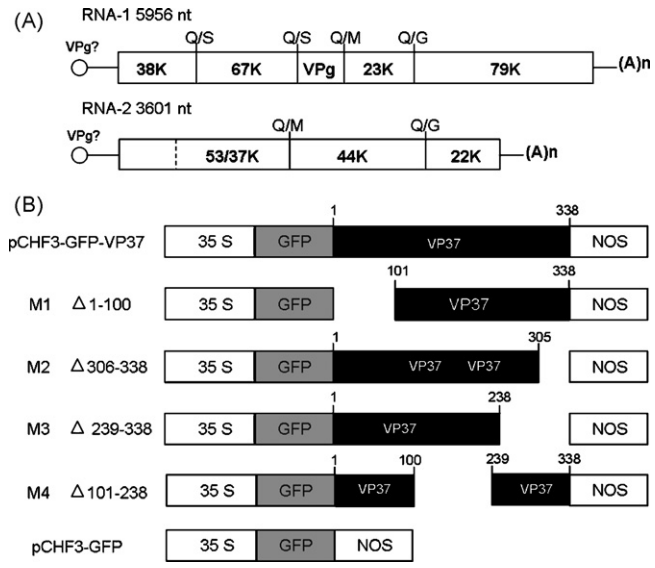

Fig. 1. Genomic organization of BBWV 2 (A), and constructions of *GFP-VP37* fusion gene and GFP-VP37 mutants in $pCHF_3$ (B). Q/S, Q/M and Q/G are cleavage sites of the polyproteins. The VP53 and VP37 proteins are C-coterminally overlapping proteins resulting from proteolytic cleavage of polyproteins from two potential translation initiation sites.

the GFP-VP37 fusion protein moves from cell to cell by itself, localizes as a halo around the nucleus and as punctate spots on the cell periphery and binds specifically to homologous viral SCP. We propose that the VP37 is a MP of BBWV 2, and the role of the protein in BBWV 2 intercellular movement is discussed.

2. Materials and methods

2.1. Construction of plasmids

Total RNA was extracted from *Chenopodium quinoa* leaves infected with BBWV 2 using Trizol reagent as instructed (Invitrogen, Carlsbad, CA, USA). Oligo(dT)-primed first strand cDNA was synthesized with the PrimeScript™ 1st Strand cDNA Synthesis Kit (Takara, Dalian, China). The BBWV 2 VP37 protein gene was amplified by PCR from cDNA using the primers VP37-BamHI-F and VP37-PstI-R (Table 1). The PCR product was cloned into the pMD-18-T vector (Takara) to produce pMD-18-T-VP37. Then, pMD-18-T-VP37 was digested with BamHI and PstI and inserted into the BamHI/PstI site of the $pCHF_3$ vector to produce $pCHF_3$-VP37 as described (Cai et al., 2007). Next, we amplified the *GFP* gene (Li et al., 2004) without the stop codon using the primers GFP-SacI-F and GFP-BamHI-R (Table 1). After digestion with SacI and BamHI restriction enzymes, the PCR product was cloned into the SacI/BamHI site within the $pCHF_3$-VP37 construct to produce plasmid $pCHF_3$-GFP-VP37. A series of *VP37* gene mutants were amplified from pMD-18-T-VP37 by appropriated primers (Table 1) to produce mutants $pCHF_3$-GFP-m1 (M1), $pCHF_3$-GFP-m2 (M2), $pCHF_3$-GFP-m3 (M3) and $pCHF_3$-GFP-m4 (M4). The M1 lacked the N-terminal 100 aa, and M2 and M3 were truncated at the C-terminal 33 or 100 aa. Overlap PCR was used to amplify *m4* gene to produce an internal deletion of aa 101–238. Using the same amplification strategy used for $pCHF_3$-VP37 and $pCHF_3$-GFP-VP37, the plasmids $pCHF_3$-GFP and $pCHF_3$-VP37-GFP were also generated.

2.2. Bombardment of Nicotiana benthamiana leaves

Leaves were detached from *N. benthamiana* (6-week-old) plants and placed in a Petri dish containing ∅9 cm qualitative filter paper. DNA constructs ($pCHF_3$-GFP and $pCHF_3$-GFP-VP37) coated microparticles were bombarded individually into the lower epidermis by using the PDS-1000/He Biolistic Delivery System (Bio-Rad, Hercules, CA, USA). Leaves were kept under moist conditions at 26 °C for 24 h and examined under confocal laser scanning microscopy (CLSM, Leica TCS SP5, Mannheim, Germany).

2.3. Agro-infiltration of N. benthamiana

Agrobacterium tumefaciens EHA105 strains containing $pCHF_3$-GFP, $pCHF_3$-GFP-VP37 or $pCHF_3$-VP37-GFP were grown overnight in YEP medium (10 g/L tryptone, 5 g/L NaCl, 10 g/L yeast extract) containing 50 μg/mL rifampicin and 50 μg/mL spectinomycin. The cultures were centrifuged and resuspended in inoculation buffer (10 mM $MgCl_2$, 10 mM MES and 100 μM acetosyringone). After incubation at 26 °C for 2 h, the culture was diluted to OD 1.0 and infiltrated into the abaxial side of a *N. benthamiana* leaf using a 1-mL syringe without a needle and infiltrated leaves were examined at 48 h post infiltration (hpi) by CLSM.

2.4. Transfection of BY-2 cells

Agrobacterium cultures harboring $pCHF_3$-GFP, $pCHF_3$-GFP-VP37, $pCHF_3$-VP37-GFP, M1, M2, M3 or M4 were washed and diluted to OD 1.0 in MS medium. BY-2 suspension cells (4 mL) were then mixed with 50 μL of *Agrobacterium* culture and after incubation at 26 °C for 48 h in darkness, the cells were washed with 3% sucrose and resuspended in MS medium. Transfected cells were kept at 26 °C in darkness and fluorescence was examined at 48 h post transfection.

2.5. Endoplasmic membrane labeling

Endoplasmic membrane (ER) labeling was performed by ER-tracker™ Blue-White DPX (Molecular Probes, Eugene, OR, USA) according to the manufacturer's protocol at a final concentration of 100 nM. BY-2 suspension cells expressing the GFP-VP37 fusion protein were stained with ER-tracker for 15 min and washed with MS medium for 3 times.

Table 1
Sequences and restriction sites of PCR primers.

Primers	Sequences (5′→3′)	Modification
Primers for constructing $pCHF_3$-GFP-X (X represents VP37 and its mutant)		
VP37-BamHI-F	CGGATCCATGAATGAGGCAAATATC	BamHI
VP37-PstI-R	GCTGCAGTTACTGGCCATACCTATAATC	PstI
GFP-SacI-F	CAGAGCTCATGGTGAGCAAGGGCGAG	SacI
GFP-BamHI-R	CGGGATCCCTTGTACAGCTCG	BamHI
VP37(N100)-F	GGATCCATGGTGCGCGCTAAAGCTG	BamHI
VP37(C100)-R	CTGCAGTTAAAATCTCTGAGAGCC	PstI
VP37(C33)-R	CTGCAGTTACTGTGCTTCACTAGC	PstI
VP37(101/238)-R	GTCGTTTTCGCTACGTCTATCTGGCAATCT	
VP37(238/101)-F	AGATTGCCAGATAGACGTAGCGAAAACGAC	
Primers for constructing $pCHF_3$-GFP and $pCHF_3$-VP37-GFP		
GFP-BamHI-F	CAGGATCCATGGTGAGCAAGGGCGAG	BamHI
GFP-PstI-R	CG CTGCAGTTACTTGTACAGCTCG	PstI
VP37-SacI-F	CCGAGCTCATGAAT GAGGCAAATATC	SacI
VP37-BamHI-R	GCGGATCCCTGGCCATACCTATAATC	BamHI
Primers for constructing pET32a-GFP, pET32a-VP37-GFP and pET32a-VP37		
VP37-SacI-F	CCGAGCTCATGAAT GAGGCAAATATC	SacI
VP37-HindIII-R	GCAAGCTTTTACTGGCCATACCTATAATC	HindIII
GFP-BamHI-F	CAGGATCCATGGTGAGCAAGGGCGAG	BamHI
GFP-HindIII-R	CG AAGCTTTTACTTGTACAGCTCG	HindIII
Primers for constructing BiFC expression vectors		
VP37-XbaI-F	GTCTAGAATGAATGAGGCAAATATC	XbaI
VP37-XhoI-R	ACTCGAGCTGGCCATACCTATAATC	XhoI
SCP-XbaI-F	ATCTAGAATGGCGCATGCGACACCC	XbaI
SCP-XhoI-R	GCTCGAGTACTTAATTGTCTTAAATATGG	XhoI