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Tutorial

Structure and components of the globular and filamentous viroplasms induced by *Rice black-streaked dwarf virus*

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ABSTRACT

Viroplasms of members of the family *Reoviridae* are considered to be viral factories for genome replication and virion assembly. Globular and filamentous phenotypes have different components and probably have different functions. We used transmission electron microscopy and electron tomography to examine the structure and components of the two viroplasm phenotypes induced by *Rice black-streaked dwarf virus* (RBSDV). Immuno-gold labeling was used to localize each of the 13 RBSDV encoded proteins as well as double-stranded RNA, host cytoskeleton actin-11 and α-tubulin. Ten of the RBSDV proteins were localized in one or both types of viroplasm. P5-1, P6 and P9-1 were localized on both viroplasm phenotypes but P5-1 was preferentially associated with filaments and P9-1 with the matrix. Structural analysis by electron tomography showed that osmiophilic granules 6–8 nm in diameter served as the fundamental unit for constructing both of the viroplasm phenotypes but were more densely packed in the filamentous phenotype.

1. Introduction

Viroplasms are highly organized structures usually of micrometer size that appear as inclusion bodies in the host cytoplasm of some virus-infected cells. They are the sites of genome replication and virion assembly (Novoa et al., 2005) and contain both viral and host factors. Many viruses induce viroplasm formation during infection, including members of the families *Herpesviridae*, *Reoviridae*, *Flaviviridae*, *Retroviridae*, *Bunyaviridae* and *Caulimoviridae* (Attoui et al., 2012; Novoa et al., 2005). Many viral proteins accumulate or are generated in the viroplasms, some of which then recruit specific cellular components (Novoa et al., 2005). For example, the viral replicase protein NS5A of *Hepatitis C virus* (HCV) modifies the host membrane to anchor its replication complex (Netherton et al., 2007), the rotavirus glycosylated phosphoprotein NSP5 recruits lipid droplets into the viroplasms where the lipid organelle may function as a replicating platform (Cheung et al., 2010; Crawford and Desselberger, 2016) and the μ2 protein of mammalian reovirus recruits and rearranges the microtubule (MT) cytoskeleton into the viroplasms to assist virion assembly (Heath et al., 2001; Parker et al., 2002). However, the function of host factors in viroplasms is still debated and some believe they not only facilitate viral replication but also take part in cellular defense against viruses (Heath et al., 2001). In this sense, viroplasms can also be considered as cytoplasmic aggresomes (Heath et al., 2001; Parker et al., 2002).

The viroplasms induced by different viruses have distinctive morphological features (Novoa et al., 2005) but viral-encoded proteins usually play important roles in their structural organization, acting as recruiters (Bienz et al., 1990) or as triggers for remodeling cellular components such as membranes or MT (Lloyd, 2001; Netherton et al., 2007; Suhy et al., 2000). Various microscopic methods have been used to probe the complexity and components of viroplasms. Confocal laser scanning microscopy (CLSM) with fluorescent markers can provide information on the behavior of the cellular components of viroplasms (Parker et al., 2002) while transmission electron microscopy (TEM) is suitable for studying the structural details (Mao et al., 2013; Wei et al., 2006b). Structural analysis by electron tomography (ET) is particularly able to pro-

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vide information of detailed structures at high resolution and in three-dimensions (3D) (Wei et al., 2009) which helps avoid image misinterpretation arising from overlapping of the details along the section thickness into a 2D projection during the imaging process (McEwen and Marko, 2001).

Reoviridae is a large virus family with members naturally infecting animals, plants and fungi (Attoui et al., 2012). Infection by animal reoviruses is mostly mild but plant reoviruses always induce severe symptoms in graminaceous hosts. Reovirus viroplasms appear either to be amorphous structures ('globular' type) filled with electron dense granular material and progeny virions (Altenburg et al., 1980; Brookes et al., 1993; Shikata and Kitagawa, 1977), or filamentous structures with progeny virions attached (Broering et al., 2002; Mao et al., 2013; Parker et al., 2002). In *Rice dwarf virus* (RDV), genus *Phytoreovirus*, as many as 10 out of a total of 12 virus-encoded proteins are associated with viroplasms (Wei et al., 2006b). The two types of viroplasms may have different components and different functions. In *Rotavirus A* (RVA), genus *Rotavirus*, the globular viroplasms are considered to be the sites of viral genome replication (Silvestri et al., 2004) while the filamentous oneshave bundles formed from MT arranged as a network (Cabral-Romero and Padilla-Noriega, 2006). Filamentous viroplasms are thought to facilitate the release of mammalian reovirus (genus *Orthovirus*) virions (Parker et al., 2002). During reoviral infection, some viruses induce only one viroplasm phenotype and some induce both. In *Southern rice black-streaked dwarf virus* (genus *Fijivirus*), a working model was proposed in which the two phenotypes sequentially functioned in genome replication and virion assembly (Mao et al., 2013) but the relationship between the two phenotypes is still far from defined.

Rice black-streaked dwarf virus (RBSDV), a recognized member of group 2 within the genus *Fijivirus*, family *Reoviridae* (Attoui et al., 2012), is a well-studied virus, causing a severe disease of rice, maize, and other cereal crops in South-East Asia. Its complete genomic sequences have been determined (Zhang et al., 2001a; Zhang et al., 2001b) and the functions of some viral genes have been assigned, among which P5-1, P6 and P9-1 have been identified to be viroplasm components (Isogai et al., 1998; Sun et al., 2013a; Wang et al., 2011; Yang et al., 2014; Zhang et al., 2008) although their distribution in the two types of viroplasm remains unclear. In infected plants, RBSDV induces cellular hypertrophy within the phloem, where the two phenotypes of viroplasm are easily accessible to TEM observation. We have examined the components and structural details of the two phenotypes of RBSDV-induced viroplasms using TEM and a series of ET analyses, with immuno-gold labeling to identify viral or host proteins.

2. Materials and methods

2.1. Infected samples and antibodies

Maize (*Zea mays* L.) plants with typical dwarf symptoms and white waxy tumors on their leaves were collected from Zhejiang province, China, and RBSDV infection was confirmed by RT-PCR (Wu et al., 2013). Polyclonal antisera against each of the 13 RBSDV-encoded proteins were produced by using recombinant proteins to immunize rabbits and their specificity verified by western blot. Antibodies against actin-11 (Agrisera, Sweden), α-tubulin (Agrisera, Sweden), and double-stranded RNA (English nm colloidal gold (Sigma, MO, USA).

2.2. Immuno-microscopy

The localization of viral encoded proteins was determined by immuno-gold labeling under the transmission electron microscope (TEM) as previously described (Xie et al., 2014) with some modification. Tissues were cut from stems into small pieces (1 × 1 mm) and fixed in a mixture of 4% paraformaldehyde and 0.1% glutaraldehyde for 4 h at 4 °C. After a 30 min treatment with 0.1 M glycerin and a series of five 30 min rinses in 100 mM phosphate buffer (pH 7.0) to remove the fixative, samples were dehydrated in a graded ethanol series (30% and 50% at 4 °C for 1 h each and 50%, 70%, 90% and 100% at −28 °C for 2 h each). They were then embedded in K4 M resin (London Inc. UK) and polymerized in ultra-violet light (360 nm) at −28 °C. Ultra-thin sections (70 nm) were cut using a Leica UC 6 microtome (Leica, Vienna, Austria) with diamond knife (Diatome, Switzerland) and placed onto 150 mesh nickel grids.

The sample grids were incubated in blocking buffer (EMS, Hatfield, PA) for 30 min at 37 °C and then in the primary antibody at 1:50 for 3 h at 30 °C. After three 5 min washes in ddH_2O, they were then incubated with secondary antibody (10 nm IgG-gold conjugates at 1:100) for 2 h at 37 °C, and washed again in ddH_2O as before. In the negative controls the primary antibody was replaced with pre-immune serum. Grids were stained with 2% uranyl acetate and 2% lead citrate for 15 min each, examined by an H-7650 TEM (Hitachi, Ibaraki, Japan) at 80 kV and photographed with a Gatan 830 CCD camera (Gatan, USA).

The density of gold labeling in regions of TEM images was determined from automatic counts and area measurements made using the 'IMARIS' software package (Bitplane, Zurich, Switzerland). Individual viroplasm modules were drawn manually and gold densities were determined from 30 viroplasms of each type. A Student's *t*-test was used to determine the significance of differences in labeling density between the non-virion matrix and the filaments.

2.3. Electron tomography and image processing

For structural observation and electron tomography (ET) analysis, small pieces (1 × 1 mm) of tissue were first fixed in 2.5% glutaraldehyde overnight and then in 1% osmium tetroxide for 4 h at room temperature. Each fixation was followed by five 30 min rinses in 100 mM phosphate buffer (pH 7.0). Samples were dehydrated in a graded ethanol series (50%, 70%, 80%, 90%, 95% and 100%) and pure acetone for 40 min each at room temperature and then infiltrated with Epon 812 resin (SPI supplies Inc. PA, USA) (30% for 3 h, 50% for 5 h, 70% for 8 h and 100% for 5 h). The resin was polymerized by gradient heating (37 °C for 12 h, 45 °C for 12 h and 60 °C for 24 h). 200 nm sections were cut using a Leica UC 6 microtome and placed onto 100 mesh carbon-covered copper grids.

After double staining with uranyl acetate and lead citrate, sections were incubated with 10 nm colloidal gold particles for 5 min which served as the fiducial markers for tomography alignments. Data were collected from a tilting specimen stage over an angular range of −60° to +60° at 1° intervals, using a FEI Tecnai F20 field emission gun (FEG) TEM (FEI, Amsterdam, Holland) at 200 kV accelerating voltage and a bottom mounted FEI TVIPS F415MP CCD (size 4k × 4k) camera with the compression parameter of 'binning = 2'. Parameters used were a defocus value of 6 μm, beam dose of 6.30 e/Å^{2}s per frame and a magnification of 50,000, and with a final image step size of 3.73 Å/pixel calculated by TEM. Tomography reconstruction was done by the IMOD 'etomo' program following the software manual. In fine alignment, we repeatedly tracked each fiducial point to the center of the gold marker until the 'residual error' value was <0.5. The final 3D tomogram was generated by a simultaneous iterative reconstruction (SIRT) algorithm in which the data calculated from the back-projection algorithm was used as the bootstrap (Kremer et al., 1996).

To analyze the distribution of granular elements (GE) in viroplasms, they were first automatically segmented by 'IMARIS v8.1' software (Bitplane, Zurich, Switzerland). To make structural comparison between the globular and filamentous phenotypes, 3D