Lettuce necrotic yellows cytorhabdovirus protein localization and interaction map, and comparison with nucleorhabdoviruses

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Received 13 September 2011
Accepted 18 December 2011

Lettuce necrotic yellows virus (LNYV), Sonchus yellow net virus (SYNV) and Potato yellow dwarf virus (PYDV) are members of the family Rhabdoviridae that infect plants. LNYV is a cytorhabdovirus that replicates in the cytoplasm, while SYNV and PYDV are nucleorhabdoviruses that replicate in the nuclei of infected cells. LNYV and SYNV share a similar genome organization with a gene order of nucleoprotein (N), phosphoprotein (P), putative movement protein (Mv), matrix protein (M), glycoprotein (G) and polymerase (L). PYDV contains an additional predicted gene of unknown function located between N and P. In order to gain insight into the associations of viral structural and non-structural proteins and the mechanisms by which they may function, we constructed protein localization and interaction maps. Subcellular localization was determined by transiently expressing the viral proteins fused to green or red fluorescent protein in leaf epidermal cells of Nicotiana benthamiana. Protein interactions were tested in planta by using bimolecular fluorescence complementation. All three viruses showed Mv to be localized at the cell periphery and the G protein to be membrane associated. Comparing the interaction maps revealed that only the N–P and M–M interactions are common to all three viruses. Associations unique to only one virus include P–Mv for LNYV, G–Mv for SYNV and M–Mv, M–G and N–M for PYDV. The cognate N–P proteins of all three viruses interacted and exhibited characteristic changes in localization when co-expressed.

INTRODUCTION

Rhabdoviruses are pathogens of vertebrates, invertebrates and plants that can have serious effects on human health, agriculture and wildlife. Taxonomically, the family Rhabdoviridae is composed of six genera, four of which infect animals and humans and replicate in the cytoplasm of infected cells. Rhabdoviruses that infect plants are assigned to two genera, Nucleorhabdovirus and Cytorhabdovirus (Dietzgen et al., 2011). Nucleorhabdoviruses replicate and assemble in the nucleus, whereas this occurs in the cytoplasm for the cytorhabdoviruses (Jackson et al., 2005). Lettuce necrotic yellows virus (LNYV) is the type species of the genus Cytorhabdovirus. In 1954, LNYV was first recognized as a destructive pathogen of lettuce (Lactuca sativa L.) causing a chlorotic and flattened appearance in the mature leaves with varying degrees of necrosis (Stubbs & Grogan, 1963). The virus forms viroplasms in the cytoplasm of infected cells and is sap transmissible from lettuce or sowthistle (Sonchus sp.) to several indicator species including Nicotiana glutinosa and petunia (Petunia × hybrid), but not to lettuce or Nicotiana benthamiana. LNYV has been reported from Australia and New Zealand, where it is transmitted in a propagative and persistent manner by the aphid vector Hyperomyzus lactucae (Dietzgen et al., 2006; Stubbs & Grogan, 1963). LNYV biological, physicochemical and genomic properties have been reviewed (Dietzgen et al., 2007). Phylogenetically, LNYV is most closely related to two other cytorhabdoviruses, Strawberry crinkle virus (SCV) and Lettuce yellow mottle virus (LYMoV) (Dietzgen et al., 2006). Sonchus yellow net virus (SYNV) and Potato yellow dwarf virus (PYDV) are members of the genus Nucleorhabdovirus with SYNV the best characterized and PYDV the type species (Jackson et al., 2005).

Rhabdoviruses are negative-sense ssRNA viruses with genomes that carry at least five genes. These genes encode a nucleoprotein (N), a phosphoprotein (P), a matrix protein (M), a glycoprotein (G) and a RNA-dependent RNA polymerase (L). The RNA, N, P and L proteins form the
viral core complex, which is the minimal infectious unit that is condensed by M proteins and surrounded by a host membrane embedded with viral glycoproteins (Jackson et al., 2005). Compared with vertebrate rhabdoviruses such as vesicular stomatitis virus (VSV), plant-adapted rhabdovirus genomes also encode an additional protein that is considered to be a putative movement protein (Jackson et al., 2005). Although the name for this protein differs depending on the virus, it will be referred to here as Mv when comparing multiple viruses and 4b when referring solely to LNYV. Some plant rhabdoviruses also encode additional accessory proteins (Walker et al., 2011), but these will not be considered here.

Studies of the protein localization and protein–protein interactions of two nucleorhabdoviruses, SYNV and PYDV, have recently been completed (Bandyopadhyay et al., 2010; Min et al., 2010) and reveal conservation among some of these interactions, but such information is not available for any cytorhabdovirus. This study focuses on LNYV: localization, co-localization and establishment of an interaction map for the viral proteins. Comparison of the interaction map for LNYV proteins to the previously published maps for nucleorhabdoviruses and a similar map for VSV (Moerdyk-Schauwecker et al., 2011) reveals that although these viruses all share a similar genome organization and virion structure, few protein interactions are shared and the maps are unique for each rhabdovirus. This suggests a possible role for host proteins in viral replication, movement and morphogenesis to bridge the gaps between viral protein interactions.

**RESULTS**

**Localization of LNYV proteins relative to the nucleus or endoplasmic reticulum (ER)**

We localized each of the viral proteins, except L, fused to both red fluorescent protein (RFP) and green fluorescent protein (GFP) in transgenic *N. benthamiana* plants expressing fluorescent markers for the nucleus and the ER. In RFP–H2B (histone 2B nuclear marker) plants, GFP fusions to LNYV proteins N, P and G localized to the cell periphery and the nuclear membranes (Fig. 1a–c, d–f, m–o). LNYV GFP–4b and GFP–M fusions co-localized with the RFP–H2B marker and were also seen outside the nucleus on the cell periphery (Fig. 1g–i, j–l). In addition, LNYV 4b and M fusions also formed small bodies in the nucleus and the cytoplasm or cell periphery that were not present in LNYV N, P or G fusions with GFP. GFP alone localized to the nucleus and cell periphery (Fig. 1p–r). In studies with LNYV proteins fused to RFP in GFP–ER plants, LNYV N, P and G co-localized with the ER membranes (Fig. 2a–c, d–f, m–o). LNYV RFP–G caused massive aggregations of the ER with areas of RFP–G accumulation not affiliated with the ER membranes. The LNYV RFP–M and RFP–4b fusions co-localized to both ER membranes and the nucleus (Fig. 2j–l, g–i), as was seen with the GFP fusions and the RFP–H2B.
To determine if the localizations of LNYV proteins are consistent with the expected sites of cytorhabdovirus replication, each protein was co-expressed in all pairwise combinations fused to either GFP or RFP. GFP–N and RFP–P co-localize in the cytoplasm to a location distinct from the localization of both N and P alone (Fig. 3a–c). GFP–N and RFP–4b co-localize to the cell periphery with some accumulation around the nucleus (Fig. 3d–f). GFP–P/RFP–M co-localize to the cell periphery, but RFP–M also localizes to the nucleus (Fig. 3g–i). GFP–4b/RFP–P co-localize to the cell periphery and the membrane around the nucleus (Fig. 3j–l). GFP–M and RFP–N co-localize to the cell periphery with some brighter accumulations of punctate loci (Fig. 3m–o). GFP–M/RFP–4b co-localize to the nucleus and cell periphery (Fig. 3p–r). GFP–G/RFP–N appeared to be associated with the ER (Fig. 4a–c). GFP–P/RFP–G localize to the cell periphery and with the most dramatic sites of co-localization at the sites where G protein accumulates (Fig. 4d–f). GFP–4b/RFP–G localized to cellular membranes; co-localization is most apparent at sites of G protein accumulation (Fig. 4g–i); this is similar to GFP–M/RFP–G localization (Fig. 4j–l). All reciprocal fusions were also tested to eliminate any affect by the auto-fluorescent protein and these localizations were identical (data not shown).

**Co-localizations of LNYV proteins**

**LNYV protein interactions in plant cells**

Bimolecular fluorescence complementation (BiFC) was done with all pairwise interactions of LNYV N, P, M, 4b and G proteins and with glutathione-S-transferase (GST) as a negative control to determine the binary interactions and localizations (Fig. 5). Four interactions were detected, M–P, M–M, N–P and P–P. An M–P interaction was detected in the cytoplasm in aggregations (Fig. 5a–c). An M–M interaction was detected inside the nucleus and on the cell periphery (Fig. 5d–f). An N–P interaction was detected outside the nucleus in aggregations similar to M–P (Fig. 5g–i). Finally, a P–P interaction was detected outside of the nucleus at the cell periphery (Fig. 5j–l). In all other pairwise BiFC combinations of LNYV proteins excluding the L protein, there were no interactions detected (data not shown). No LNYV proteins tested interacted with GST in either orientation (Fig. 5m–o and data not shown).

**Comparison of LNYV protein interactions to those of other plant-adapted rhabdoviruses**

Plant rhabdovirus protein–protein interactions have not previously been compared to determine if there are similarities between them. Here, three viruses were compared for their similarities in BiFC interaction assays, the cytorhabdovirus LNYV and the nucleorhabdoviruses PYDV and SYNV. A positive interaction was detected for all cognate N and P proteins for LNYV, PYDV and SYNV. LNYV N–P is localized outside the nucleus (Fig. 6a–c). PYDV N–P is
localized inside the nucleus (Fig. 6d–f), and so is SYNV N–P (Fig. 6g–i). All three viruses also share an M–M interaction. LNYV M–M is localized in the nucleus and on the cell periphery (Fig. 6j–l). PYDV M–M and SYNV M–M are both localized inside the nucleus (Fig. 6m–o, PYDV; Fig. 6p–r, SYNV).

**DISCUSSION**

Even before the advent of nucleic acid sequencing, rhabdoviruses in plants were classified into two genera, *Nucleorhabdovirus* and *Cytorhabdovirus*, based on serology, electron micrographs of the cell and particle morphology.
As genome sequencing of these viruses progressed, the distinction of viruses into the two genera has been maintained (Bandyopadhyay et al., 2010; Dietzgen et al., 2006; Ghosh et al., 2008; Redinbaugh et al., 2002). The genome sequence for LNYV became publicly available in 2006 and represents the second cytorhabdovirus to be completely sequenced after Northern cereal mosaic virus (Dietzgen et al., 2006; Tanno et al., 2000). LNYV is most closely related to SCV and LYMv based on the phylogeny constructed from the polymerase motif of the L gene sequences (Dietzgen et al., 2007; Heim et al., 2008). The entire sequence of SCV is not available for comparison although it has been reported as completed (Schoen et al., 2004). No protein localizations are known for any of the cytorhabdoviruses (Heim et al., 2008; Tanno et al., 2000). This is the first report of the localization and interactions of proteins from a cytorhabdovirus and facilitates for the first time a comparison between cytorhabdovirus and nucleorhabdoviruses for those characteristics. PYDV and SYNV are nucleorhabdoviruses that have previously been shown to induce differential nuclear morphology during viral morphogenesis (Goodin et al., 2005).

All LNYV proteins localize to the cytoplasm on the cell periphery, and 4b and M proteins also localize inside the nucleus. All proteins co-localize with the ER marker in transgenic plants. This is consistent with the localization of the virion in electron micrographs (Chambers et al., 1965). Cytorhabdoviruses, unlike nucleorhabdoviruses, do not have an intimate association with the nucleus and nuclear membranes. The current model for cytorhabdovirus replication begins with the entry of a virion into the cell, uncoating in association with ER membranes to release the viral core, synthesis of viral mRNAs which leads to the synthesis of viral proteins, and the formation of a viroplasm in the cytoplasm (presumably near or on the ER membranes) that leads to budding of the mature virions through the ER membranes (Jackson et al., 2005). The localization of each of the LNYV proteins to the ER agree with this model of replication. The partial nuclear localization of 4b and M is unexpected and the role of these proteins in the nucleus is unknown. In the animal rhabdovirus VSV, which also replicates in the cytoplasm, the M protein localizes to the nuclear rim to block export of host mRNAs in the infected cells through interactions with nuclear export proteins (Faria et al., 2005) and has been proposed as a means of limiting competition of resources for the viral proteins (Faria et al., 2005; von Kobbe et al., 2000). There may be a similar reason for the localization of LNYV M in the nuclear periphery, but tests to determine the binding of M protein to nuclear export factors have not been done to determine this. The putative LNYV Mv 4b is also present in the nucleus, and this localization of 4b and M may also be linked to viral movement, possibly in the recruitment of host transcription factors as seen for SYNV (Min et al., 2010). In the nucleorhabdoviruses SYNV and PYDV, the M localize to the cell periphery, but both M proteins localize to the nucleus and are considered to be part of a movement complex (Bandyopadhyay et al., 2010; Goodin et al., 2007; Min et al., 2010).

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Previous studies of LNYV-infected N. glutinosa using electron microscopy showed viral particles in the ER of infected cells, never in the nucleus, though particles were
seen in the ER closely adjoining the nucleus (Chambers et al., 1965). While principally replicating in association with cytoplasmic viroplasms, LNYV multiplication appears to have an early ‘nuclear phase’ 5–7 days after infection, which is characterized by cellular changes in the nucleus.

Electron microscopic examination of the outer nuclear membrane of infected cells identified ‘blisters’ containing small vesicles at the time when first symptoms appeared (Wolanski & Chambers, 1971). This observed cytopathology may be due to the presence of M and/or 4b proteins in the nucleus.

The co-localizations of LNYV proteins agree with the localizations of the individual proteins, with the exception of N–P. When LNYV N and P are co-expressed they localize to aggregate formations outside the nucleus. This is similar to the nucleorhabdoviruses SYNV, PYDV and Maize fine streak virus (MFSV), where N–P co-localize to subnuclear loci distinctly different from the localization of either protein alone (Bandyopadhyay et al., 2010; Goodin et al., 2001; Tsai et al., 2005). An N–P interaction is also observed in VSV (Takacs & Banerjee, 1995; Takacs et al., 1993), and may represent a conserved interaction and localization pattern in all rhabdoviruses. The LNYV co-localizations were done in cells of uninfected N. benthamiana plants and may be affected by the presence of replicating virus. Some changes in the localization of the proteins of SYNV were noticed when examined in virus-infected cells (Goodin et al., 2007). Using the SYNV experience as an example, the viroplasm may be more identifiable studying LNYV protein co-localizations in the context of infection. Such experiments would need to be done in another related plant species, N. glutinosa, which unlike N. benthamiana is a systemic host for LNYV (Dietzgen et al., 2007).

BiFC offers the advantage of localization, interaction and comparison to other rhabdoviruses previously tested (Bandyopadhyay et al., 2010; Citovsky et al., 2006; Martin et al., 2009; Min et al., 2010). When testing protein–protein interactions of LNYV by BiFC, only four interactions were detected, N–P, P–P, M–P and M–M. Compared with SYNV and PYDV, the only two plant rhabdoviruses whose interaction maps have been completed, this is the fewest number of interactions detected (Fig. 7). The N–P interaction of LNYV is similar to the N–P co-localization observed with aggregations outside the nucleus (Fig. 3). The P–P self-interaction is similar to that observed for SYNV and PYDV, as it resembles the single expression of P.
The M–M self-interaction is conserved in SYNV, PYDV and LNYV (Fig. 7). It has also been described for the animal rhabdoviruses, VSV and *Lagos bat virus* (Ge et al., 2010; Graham et al., 2008). Unlike SYNV, PYDV and VSV, LNYV has no detectable G–G interaction. In VSV, the G–G self-interaction is characterized and the protein is part of a homotrimer (Roche et al., 2006, 2008). It was unexpected that G protein self-interactions were not seen for LNYV. It might be that the yellow fluorescent protein (YFP) halves in the BiFC assay may not be able to come together due to steric constraints. In all our BiFC assays, YFP halves were attached to the amino-termini of the proteins. When the YFP half was fused to the carboxy terminus of the G protein, this did not result in a detectable G–G interaction either (data not shown). This does not rule out the possibility that such an interaction occurs that may be detected in alternative assays such as yeast-two-hybrid or pull-down. Only the N–P and M–M interactions are conserved among the plant rhabdoviruses studied and VSV, and other interactions do not appear to be conserved (Fig. 7).

We tested the interactions of LNYV proteins in the non-host *N. benthamiana*, due to the ease of infiltration and, importantly, when the same interactions were tested in the host plant lettuce, no discernible differences were observed other than a decrease in the amount of aggregations present in the P–P interaction (data not shown). This suggests that the viral protein interactions seen in *N. benthamiana* represent the interactions in lettuce as well. Also, since the experiments were done in a non-host plant, which permits neither replication in inoculated leaves nor systemic movement of viruses throughout the plant, these interactions were not tested in the context of virus infection. Lettuce cannot be mechanically inoculated with LNYV and agroinfiltration of this species is tedious. It may be possible that the presence of replicating virus is required for some interactions to occur. This was the case for the VSV M–N interaction, which does not occur unless the cell is infected. Moerdyk-Schauwecker *et al.* (2009) hypothesized that interaction during infection was detected because either the presence of multiple viral proteins is needed or there is host protein recruitment (Flood & Lyles, 1999; Lyles & McKenzie, 1998). VSV also incorporates a number of host proteins into the virion during assembly and these may be responsible for bridging the gaps that are seen even between the proteins in a VSV interaction map (Moerdyk-Schauwecker *et al.*, 2011). When plant host factors are added to the SYNV interaction map, the gaps between some viral proteins are bridged (Min *et al.*, 2010). It is expected that this will also be the case for LNYV.

This is the first report of protein localization and interactions for a cytorhabdovirus. The protein localizations and interactions are very different from those of the previously described nucleorhabdoviruses PYDV, SYNV and MFSV. The localization of LNYV proteins most closely resembles that of the animal rhabdovirus model VSV but there are several differences in this comparison as well (Fig. 7). Although both viruses replicate in the cytoplasm, VSV buds from the plasma membrane, but LNYV buds into the ER. This study clearly demonstrates that although the rhabdoviruses may have analogous genes, gene order and virus structure, there are clear differences in the protein interactions that occur.

**METHODS**

**Plant growth.** Wild-type and transgenic *N. benthamiana* plants expressing fluorescent markers targeted to the ER and nucleus were maintained in a greenhouse under ambient conditions (Martin *et al.*, 2009).

**Protein expression in *N. benthamiana*.** Viral sequences corresponding to all known ORFs of LNYV except L were amplified by RT-PCR using Phusion proofreading DNA polymerase (Invitrogen), cloned into pDONR221 using the Gateway cloning system (Invitrogen) and sequenced. The LNYV sequences (GenBank accession NC_007642) used for amplification were from the fully sequenced isolate 318, an Australian isolate found originally in garlic (Dietzgen *et al.*, 2006, 2007). The pDONR clones were then recombined into the binary destination vectors for expression of autofluorescent protein fusions in plant cells as described previously (Chakrabarty *et al.*, 2007; Goodin *et al.*, 2007; Martin *et al.*, 2009). Vectors utilized in this study were pSITE-2CA (GFP fusions), pSITE-4CA (mRFP fusions) for localization experiments and pSITE-nEYFP-C1 and pSITE-cEYFP-C1 for BiFC experiments. Recombinant vectors containing the gene of interest were transformed into *Agrobacterium tumefaciens* strain LBA4404. Agroinfiltration for expression of protein fusions in plant cells was conducted as described previously (Goodin *et al.*, 2005). BiFC analyses were done in transgenic *N. benthamiana* expressing a cyan fluorescent histone 2B protein (CFP–H2B) for simultaneous localization of the nucleus. Each expression construct was examined in a minimum of three leaves from three plants in independent
experiments and at least three high-quality images were acquired for each construct. BiFC assays were done as described for the production of protein interaction maps for PYDV and SYNV (Bandyopadhyay et al., 2010; Min et al., 2010). LNYV protein BiFC interactions were compared with images from SYNV and PYDV protein interactions similar to those previously published (Bandyopadhyay et al., 2010; Min et al., 2010).

**Laser scanning confocal microscopy.** All microscopy was done on an Olympus FV1000 laser scanning confocal microscope 2 days post-infiltration as described previously (Goodin et al., 2005). BiFC analyses were done as described previously (Bandyopadhyay et al., 2010; Min et al., 2010). All proteins were tested as carboxy-terminal fusions to the amino (nec) or carboxy (cec) terminal portions of YFP. Fusions with glutathione-S-transferase (GST) served as a negative binding control.

**ACKNOWLEDGEMENTS**

We thank Ms Kirstin Kopperud, Ms Carol Seiler and Mr Roger Mitchell for technical assistance. Funding by the National Science Foundation (to M. M. G.) and the State of Queensland through its Department of Employment, Economic Development and Innovation (DEEDI; to R. G. D.) is gratefully acknowledged. Visits to the University of Kentucky by R. G. D. were supported by a DEEDI Queensland International Fellowship (2010) and by an international linkage grant from the Australian Academy of Science (2007).

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