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Cytorhabdovirus P3 genes encode 30K-like cell-to-cell movement proteins

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ABSTRACT

Plant viruses encode movement proteins (MP) to facilitate cell-to-cell transport through plasmodesmata. In this study, using *trans*-complementation of a movement-defective turnip vein-clearing tobamovirus (TVCV) replicon, we show for the first time for cytorhabdoviruses (lettuce necrotic yellows virus (LNYV) and alfalfa dwarf virus (ADV)) that their P3 proteins function as MP similar to the TVCV P30 protein. All three MP localized to plasmodesmata when ectopically expressed. In addition, we show that these MP belong to the 30K superfamily since movement was inhibited by mutation of an aspartic acid residue in the critical 30K-specific LxD/N_{50-70}G motif. We also report that *Nicotiana benthamiana* microtubule-associated VOZ1-like transcriptional activator interacts with LNYV P3 and TVCV P30 but not with ADV P3 or any of the MP point mutants. This host protein, which is known to interact with P3 of sonchus yellow net nucleorhabdovirus, may be involved in aiding the cell-to-cell movement of LNYV and TVCV.

Introduction

The local spread of viruses in plants is mediated by movement proteins (MP), which ferry infectious genetic material from cell-to-cell through the modification of membrane-lined pores in the cell wall referred to as plasmodesmata (PD) (Ding et al., 1992; Robards and Lucas, 1990; Sevilem et al., 2013). This is exemplified by tobacco mosaic virus (TMV) P30 MP, which was shown to increase PD pore size by nearly 10-fold (Wolf et al., 1989). Following cell-to-cell spread, MP assist in the transport of the viral genome to vascular (phloem) tissues, which in susceptible species leads to systemic spread of virus-like particles (VLPs) and/or virions into the rest of the plant (Ryabov et al., 1998; Scholthof, 2005).

The 30K MP superfamily was recently proposed to be one of the most phylogenetically diverse plant virus gene families (Mushegian and Elena, 2015). These specialized plant viral MP have been shown to similarly facilitate cell-to-cell movement by binding to nucleic acids, localizing to the cell periphery at PD, and increasing PD pore size (Citovsky et al., 1990; Koonin et al., 1991; Melcher, 2000; Oparka et al., 1997; Tomenius et al., 1987; Wolf et al., 1989). In addition, some members of this MP superfamily such as tomato spotted wilt tospovirus NSm can form tubules through PD to facilitate cell-to-cell spread of VLPs (Amari et al., 2010; Kasteel et al., 1996; Storms et al., 1995). Despite a lack of amino acid sequence conservation, one of the defining structural characteristics of 30K-like MP is a common core domain (CCD) that consists of two to four α-helices that flank multiple β-strands (Melcher, 2000). Recent studies have proposed that mutating the D residue in the LxD/N_{50-70}G motif in the 30K CCD can be used to functionally validate that a MP belongs to the 30K superfamily (Mushegian and Elena, 2015; Yu et al., 2013).

Plant-infecting rhabdoviruses (genera *Cytorhabdovirus* and *Nucleorhabdovirus*) unlike their animal- and human-infecting counterparts, are also thought to encode MP, some of which have been predicted to be 30K-like based on similar secondary structure to that of known members of the 30K-like superfamily (Dietzgen et al., 2006; Huang et al., 2005; Melcher, 2000). The rhabdoviral 30K-like MP superfamily candidates predicted by secondary structure are sonchus yellow net virus (SYNV) sc4 and lettuce necrotic yellows virus (LNYV) 4b (Dietzgen et al., 2006; Jackson et al., 2005; Melcher, 2000). LNYV 4b also showed limited amino acid sequence similarities with 30K MP superfamily members, capilloviruses and trichoviruses of the *Betaflexiviridae* family (Dietzgen et al., 2006). In addition, plant rhabdovirus putative MP genes have also been predicted by genome location. This is mostly as the third gene from the 3′ end of the genome between the phosphoprotein (P) and matrix protein (M) genes as

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for example, alfalfa dwarf virus (ADV) P3, lettuce yellow mottle virus (LYMoV) P3, maize mosaic virus (MMV) P3, maize Iranian mosaic virus P3, maize fine streak virus (MFSV) P4, rice yellow stunt virus (RYSV) P3, potato yellow dwarf virus (PYDV) Y protein, northern cereal mosaic virus (NCMV) P3, and taro vein chlorosis virus (TaVCV) P3 (Bandyopadhyay et al., 2010; Bejerman et al., 2015; Heim et al., 2008; Hiraguri et al., 2010; Huang et al., 2005; Massah et al., 2008; Reed et al., 2005; Revill et al., 2005; Scholthof et al., 1994; Tanno et al., 2000; Tsai et al., 2005). However, to date MP function has been experimentally demonstrated for P3 of only a single plant rhabdovirus. The nucleorhabdovirus RYSV P3 was shown to *trans*-complement the movement function of a movement-defective potato virus X (PVX) in *Nicotiana benthamiana* (Huang et al., 2005). Similarly, P3 of rice transitory yellowing virus (RTYV), a strain of RYSV, was also shown to function as MP (Hiraguri et al., 2012). Indirect evidence supporting a role for plant rhabdovirus P3 in cell-to-cell movement has been provided by autofluorescent protein fusions of SYNV sc4, PYDV Y, MFSV P4, ADV P3, and LNYV 4b which all localized to the cell periphery (Bandyopadhyay et al., 2010; Bejerman et al., 2015; Dietzgen et al., 2012; Goodin et al., 2007b; Tsai et al., 2005). A more in-depth investigation proposed a model for nucleorhabdovirus SYNV cell-to-cell movement and showed that two predominantly microtubule-associated *N. benthamiana* proteins, sc4 interactor protein 17 (sc4i17; motor kinesin homolog) and sc4i21 (*Arabidopsis thaliana* vascular one-zinc finger protein 1 homolog; AtVOZ1) (Mitsuda et al., 2004) interact with SYNV sc4 protein in a multi-protein complex that may aid cell-to-cell movement (Min et al., 2010). Using the first reverse genetics system for a plant negative-strand RNA virus, researchers have shown recently that sc4 protein is required for SYNV cell-to-cell movement (Wang et al., 2015).

Cell-to-cell movement function of a viral protein is typically tested by co-expressing a putative MP in *trans* with a movement-defective virus. In this assay, the movement-defective virus construct encodes green fluorescent protein (GFP), which permits a visual evaluation of cell-to-cell spread (Agranovsky et al., 1998; Ajjikuttira et al., 2005; Huang et al., 2005; Lauber et al., 1998; Lewandowski and Adkins, 2005; Li et al., 2004; Morozov et al., 1997; Ryabov et al., 1998; Solovyev et al., 1996; Zhang et al., 2005). Experimental validation of putative MP function has frequently employed tobamovirus- or PVX-based movement complementation systems (Hiraguri et al., 2012; Ishikawa et al., 2013; Niehl et al., 2014; Yu et al., 2013) and 30K-like MP have been shown to work in both (Lauber et al., 1998; Solovyev et al., 1996; Ziegler-Graff et al., 1991).

In this study, we tested movement function of two cytorhabdovirus putative MP using a movement-defective version of a turnip vein-clearing tobamovirus (TVCV) pro-vector system (Giritch et al., 2006). The tobamovirus pro-vector system was originally developed for commercial scale expression of a gene/protein of interest in plants using a modified TVCV vector that had been engineered for high-level replication by introducing multiple introns to make it more "plant-like" (Marillonnet et al., 2005). This pro-vector system is delivered by agroinfiltration into *N. benthamiana* leaves as a mixture consisting of three individual viral gene modules that assemble *in planta* to form a single infectious viral replicon (Giritch et al., 2006; Marillonnet et al., 2004). The three modules are: i) 5′ module encoding TVCV RNA-dependent RNA polymerase and TVCV P30 MP (pICH-17388), ii) 3′ module encoding GFP reporter and carrying a crucifer-infecting TMV 3′ NTR (pICH-7410) and iii) integrase module that combines the 5′ and 3′ modules into a single replicon through phiC31-dependent homologous recombination (pICH-14011). In the pICH-7410 module, the viral capsid protein was replaced by GFP rendering the replicon incapable of systemic spread but still maintaining efficient replication and cell-to-cell movement, which is indirectly visualized by GFP expression (Giritch et al., 2006).

Functional MP studies have yet to be conducted for any cytorhabdovirus. To continue our characterization of cytorhabdovirus-encoded proteins (Bejerman et al., 2015; Mann et al., 2015; Martin et al., 2012), we set out to experimentally validate the predicted cell-to-cell movement function of P3 of the cytorhabdoviruses, LNYV and ADV. Both viruses have a similar conserved 3′→5′ basic genome organization, nucleoprotein (N) – P – P3 – M – glycoprotein (G) – polymerase (L). However, ADV encodes an additional P6 ORF between G and L genes (Bejerman et al., 2015), similar to strawberry crinkle cytorhabdovirus (Mann and Dietzgen, 2014; Schoen et al., 2004). The predicted MP ORF for LNYV and ADV is at genome position 3 between P and M genes (referred to as P3). However, LNYV putative MP has been historically referred to as "4b", based on the relative size of the respective viral mRNA in northern blots (Dietzgen et al., 1989). We will refer here to both cytorhabdoviral MP as "P3". In this study, we demonstrate that LNYV P3 and ADV P3 function as MP by supporting the movement of a P30-defective TVCV replicon in *trans*. We also perform mutation analysis of the crucial 30K-like LxD/N_{50-70}G motif that is present in both of these MP and examine P3 interaction with a microtubule-associated host protein to provide further insights into the cell-to-cell movement process of these 30K-like MP.

Results

Comparison of putative plant rhabdovirus movement protein sequences with those of confirmed 30K-like MP superfamily members

Secondary structure predictions for putative MP of some plant rhabdoviruses, including LNYV P3, have revealed 30K-like MP sequence features (Melcher, 2000; Walker et al., 2011). To explore this in more detail we conducted sequence homology analyses for both LNYV P3 and the previously uncharacterized ADV P3 using the National Center for Biotechnology Information non-redundant database. BlastP searches identified a 30K-like MP domain in LNYV P3 between amino acids 40 and 185, but no such domain was found in ADV P3 sequence.

We next investigated whether LNYV P3 and ADV P3 shared any secondary structure homology with 30K superfamily tobamovirus members TMV and TVCV. Alignments based on both sequence and secondary structure similarities were carried out using PROMALS3D (Pei and Grishin, 2014). With respect to the 30K-specific CCD, both LNYV P3 and ADV P3 possessed a secondary structure pattern similar to that reported for members of the 30K superfamily. The consensus pattern of the CCD for 30K MP members consists of four α-helices (αA–αD) interspersed by seven β-elements (β1–β7) (Fig. 1A) (Melcher, 2000). However, for the cytorhabdovirus P3's, an additional β-element was observed after β7, named β7′ (Fig. 1A). Two of the conserved residues in the CCD, namely aspartic acid (D) and glycine (G) (Fig. 1A; black boxes) in the cytorhabdovirus P3 conform to the 30K-specific LxD/N_{50-70}G motif. This motif which starts at the end of the β2 sheet in what is called the 30K domain (Fig. 1A; second black box), is conserved for most 30K MP members (Melcher, 2000) and mutation of the D residue has been shown to cause the disruption of both, movement function and localization to PD for several 30K members (Bertens et al., 2000; Li et al., 2009; Yu et al., 2013; Zhang et al., 2012). An additional PROMALS3D alignment to test whether this motif is present in other plant rhabdovirus P3 sequences found that all available P3 protein sequences of cytorhabdoviruses (ADV, barley yellow striate mosaic virus, LNYV, LYMoV, persimmon virus A and NCMV) contained the D residue of the LxD/N_{50-70}G motif, whereas dichorhaviruses (orchid fleck virus and coffee ringspot