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Movement and nucleocapsid proteins coded by two tospovirus species interact through multiple binding regions in mixed infections

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ABSTRACT

Negative-stranded tospoviruses (family: *Bunyaviridae*) are among the most agronomically important viruses. Some of the tospoviruses are known to exist as mixed infections in the same host plant. *Iris yellow spot virus* (IYSV) and *Tomato spotted wilt virus* (TSWV) were used to study virus–virus interaction in dually infected host plants. Viral genes of both viruses were separately cloned into binary pSITE-BiFC vectors. BiFC results showed that the N and NSm proteins of IYSV interact with their counterparts coded by TSWV in dually infected *Nicotiana benthamiana* plants. BiFC results were further confirmed by pull down and yeast-2-hybrid (Y2H) assays. Interacting regions of the N and NSm proteins were also identified by Y2H system and β-galactosidase activity. Several regions of the N and NSm were found interacting with each other. The regions involved in these interactions are presumed to be critical for the functioning of the tospovirus N and NSm proteins. This is the first report of *in vivo* protein interactions of distinct tospoviruses in mixed infection.

Introduction

Multiple interactions between plant viruses are quite common in nature (DaPalma et al., 2010; Hisa et al., 2014; Matthews, 1991). Multiple/mixed infection also facilitates the genetic reassortment among different virus species. The possible outcomes of these interactions can be elimination of one virus from the mixed infection by the second virus, co-existence of both viruses for the remainder of the plant's life, increased titer of one virus resulting in more severe symptoms (synergism), or expansion of the host range (Hammond et al., 1999; Hisa et al., 2014; Syller, 2012; Wege and Siegmund, 2007). Cross protection and mutual exclusion have been observed in antagonistic interactions between closely related viruses. Facilitative interactions between distinct viruses have been shown to cause synergism, where one virus enhances the virulence or complements the defects of the other one and helps it in replication, systemic movement or transmission. This could lead to more severe disease and may increase crop damage and yield losses (Hisa et al., 2014). Synergism can be of different natures-mutual-when both viruses increase in levels, unilateral-when only one virus increases in concentration, and neutral-when the virus concentration does not change (Zhang et al., 2001). Synergism has been reported across several genera of plant viruses. Synergistic interactions in plant viruses were best studied in *Potyviridae* family between *Potato virus Y* (PVY, genus *Potyvirus*) and *Potato virus X* (PVX, genus *Potexvirus*). PVY and PVX have been shown to interact with a number of related and unrelated potyviruses (Reviewed in Hisa et al., 2014). However, there is limited or no information available on synergistic interactions of tospoviruses. Synergistic interactions are also important for understanding the potential for reassortment between tospoviruses. Natural reassortment between tospoviruses, *Groundnut ringspot virus* (GRSV) and *Tomato chlorotic spot virus* (TCSV), has been reported recently (Webster et al., 2011). In addition to that, several reassortment patterns were observed in *Tomato spotted wilt virus* (TSWV) populations by phylogenetic studies suggesting mixed tospovirus infections in nature (Tentchev et al., 2011).

Tospoviruses belong to the negative-stranded *Bunyaviridae* family and are among the most agronomically important viruses (Scholthof et al., 2011). Of the five genera in family *Bunyaviridae*, members of the genus *Tospovirus* are the only viruses that infect plants (Pappu et al., 2009). The host range of the type member of tospoviruses, TSWV, includes 1090 plant species in 15 families of monocotyledonous and 69 families of dicotyledonous plants worldwide (Li et al., 2009). TSWV causes chlorosis, necrosis, and ringspots on leaves, fruits and stems of the host plants and infection often leads to wilting and death of plants (Li et al., 2009, Parrella et al., 2003).

Since the discovery of TSWV, more than 30 distinct tospoviruses have been reported worldwide Mandal et al., 2012. In addition to TSWV, *Iris yellow spot virus* (IYSV) is another important tospovirus in the US. First reported by Hall et al. (1993) in onion crop, IYSV has now a wide occurrence in several states of the

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U.S and many parts of the world (Hall et al., 1993). IYSV could cause complete crop loss in onions, especially in seed crops (Pappu et al., 2009).

The genome of tospoviruses consists of three RNAs, large (L), medium (M) and small (S). The L RNA is organized in negative sense orientation, whereas the M and S RNAs are in ambisense (Adkins, 2000). The L RNA codes for the RNA dependent RNA polymerase (RdRp) in the negative sense, the M RNA codes for a nonstructural protein, NSm, in sense direction and the glycoprotein precursor (G_N/G_C) in antisense orientation. The S RNA codes for a non-structural protein (NSs) in sense direction and the nucleocapsid protein (N) in antisense direction. The NSm and G_N/G_C proteins coded by the M RNA, play important roles in cell-to-cell movement in the host and in vector transmission, respectively (Adkins, 2000; Kormelink et al., 1994; Whitefield et al., 2005). The nucleocapsid (N) protein and silencing suppressor (NSs) protein are coded by the S RNA (Adkins, 2000; Pappu, 2008; Takeda et al., 2002; Tsompana and Moyer, 2008).

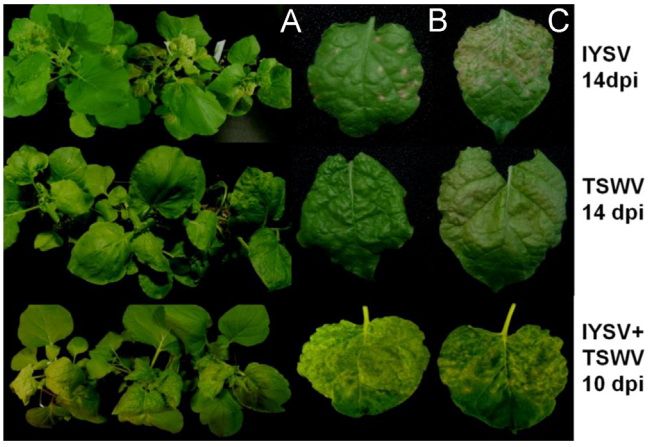

Fig. 1. Symptoms of *Iris yellow spot virus* (IYSV) and *Tomato spotted wilt virus* (TSWV) infections on *Nicotiana benthamiana*. Whole plants (A), inoculated leaves (B), and systemic leaves (C). Symptoms shown were at 10–14 days after IYSV, TSWV or IYSV+TSWV inoculations.

Multiple functions of TSWV N have been reported in several studies. The N protein contains multiple RNA binding domains through which it binds nonspecifically with ssRNA (Richmond et al., 1998). Homotypic interaction and multimerization of the N protein has been speculated as prerequisite for packaging and protecting the viral genome (Uhrig et al., 1999).

TSWV NSm is expressed during a short period early in systemic infection. It also associates with N, ssRNA, and host proteins (Li et al., 2009). NSm's interaction with N protein has been identified as a primary requirement for cell–cell movement and long distance movement. Moreover, TSWV NSm domains required for tubule formation, movement and symptom development have been identified (Li et al., 2009). Phylogeny based on nucleocapsid (N) and movement (NSm) proteins has grouped IYSV with "Old World" or Eurasian tospovirus species, while TSWV with the "new world" or the American species of tospoviruses. Similarity between N and NSm proteins of IYSV and TSWV is approximately 33% and 36%, respectively (Chiemsombat and Adkins, 2006; Silva et al., 2001; Uhrig et al., 1999).

Mixed infections of two distinct tospoviruses in the same host plant have been reported to occur in commercial production

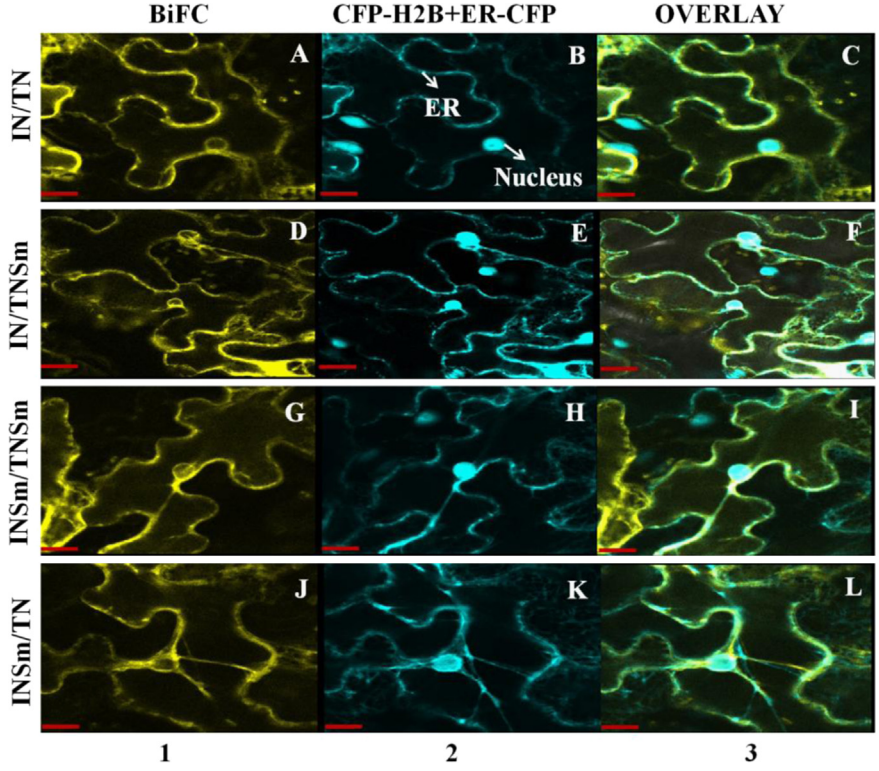

Fig. 2. *In planta* interaction of *Iris yellow spot* and *Tomato spotted wilt* virus nucleocapsid (N) and movement (NSm) proteins interactions examined by BiFC. Interaction assays were performed in leaf epidermal cells of IYSV+TSWV-infected transgenic *Nicotiana benthamiana* plants expressing cyan fluorescent protein fused to the nuclear marker histone 2B (CFP-H2B), and cyan endoplasmic reticulum (ER-CFP) marker. Column 1 shows BiFC, column 2 shows localization of CFP-H2B and ER-CFP (nucleus and ER), and column 3 shows a merge of all panels (overlay). The first and second proteins mentioned in each pair of interactors were expressed as C-terminal fusions to the amino-terminal half of YFP and as C-terminal fusions to the carboxy-terminal half of YFP respectively. A set of positive interactions is shown here after testing interactions in all pairwise combinations: (A–C) IYSV N/TSWV N, (D–F) IYSV N/TSWV NSm, (G–I) IYSV NSm/TSWV NSm and (J–L) IYSV NSm/TSWV N. Micrographs shown are representative of at least 50 cells examined. Scale bar = 20 μm.

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