Tospoviruses cause economically significant losses in diverse cropping systems globally (Pappu et al., 2009). They are transmitted by thrips in a propagative manner. Taxonomically, tospoviruses have been classified in the genus Tospovirus, family Bunyaviridae. The type species and best-studied tospovirus is Tomato spotted wilt virus (TSWV) after which the genus is named (Kormelink et al., 2011). Tospoviruses have a tripartite negative- and ambi-sense RNA genome encodes six proteins that are involved in cytoplasmic replication, movement, assembly, insect transmission and defence. To gain insight into the associations of these viral proteins, we determined their intracellular localization and interactions in living plant cells. Nucleotide sequences encoding the nucleoprotein N, non-structural proteins NSs and NSm, and glycoproteins Gn and Gc of a Kentucky isolate of INSV were amplified by RT-PCR, cloned, sequenced and transiently expressed as fusions with autofluorescent proteins in leaf epidermal cells of Nicotiana benthamiana. All proteins accumulated at the cell periphery and co-localized with an endoplasmic reticulum marker. The Gc protein fusion also localized to the nucleus. N and NSm protein self-interactions and an NSm–N interaction were observed by using bimolecular fluorescence complementation. A tospovirus NSm homotypic interaction had not been reported previously.

A Kentucky isolate of INSV from glasshouse-grown Arabidopsis thaliana (INSV-K) was passaged twice in Nicotiana benthamiana and infected leaves stored at −80 °C. Total RNA was extracted using RNeasy Plant Mini kit (Qiagen) and cDNA generated using Superscript III reverse transcriptase (Invitrogen) and oligo(dT)12–18 or random hexamer primers. INSV ORFs for the N, NSs, NSm, Gn and Gc proteins were amplified by PCR using Phusion high-fidelity DNA polymerase (Finnzymes) and attB sequence-flanked virus-specific primers (Table S1, available in JGV Online). INSV-specific primer sequences were designed from the published sequences of the L, M and S RNAs of an INSV isolate (GenBank accession nos GQ336989–GQ336991) from Phalaenopsis orchid from Yunnan, China (Cheng et al., 2010). Amplicons were cloned into pDONR221 (Invitrogen) and two to three clones each sequenced in both directions using M13 forward and reverse primers and internal sequencing primers as required (Table S1). Sizes of the ORFs within the gene sequences of INSV-K used in this study were 786 (N), 1341 (NSs), 909 (NSm), 1395 (Gn) and 2019 nt (Gc). GenBank accession numbers for the N, NSs, NSm and G precursor (Gn plus Gc) coding sequences are JX138531, JX138533, JX138532, JX138530, respectively.

Protein expression in N. benthamiana cells for protein localization or bimolecular fluorescence complementation including orchids, fuchsia, begonia, dahlia and chrysanthemum (Daughtrey et al., 1997). Phylogenetically, INSV belongs to the ‘Americas’ clade of tospoviruses together with TSWV (Pappu et al., 2009).

In planta localization and interactions of impatiens necrotic spot tospovirus proteins

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Impatiens necrotic spot tospovirus (INSV) is a significant pathogen of ornamentals. The tripartite negative- and ambi-sense RNA genome encodes six proteins that are involved in cytoplasmic replication, movement, assembly, insect transmission and defence. To gain insight into the associations of these viral proteins, we determined their intracellular localization and interactions in living plant cells. Nucleotide sequences encoding the nucleoprotein N, non-structural proteins NSs and NSm, and glycoproteins Gn and Gc of a Kentucky isolate of INSV were amplified by RT-PCR, cloned, sequenced and transiently expressed as fusions with autofluorescent proteins in leaf epidermal cells of Nicotiana benthamiana. All proteins accumulated at the cell periphery and co-localized with an endoplasmic reticulum marker. The Gc protein fusion also localized to the nucleus. N and NSm protein self-interactions and an NSm–N interaction were observed by using bimolecular fluorescence complementation. A tospovirus NSm homotypic interaction had not been reported previously.

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(BiFC; Citovsky et al., 2008) was done essentially as described by Martin et al. (2009). Briefly, sequence-validated full-length ORF entry clones without a stop codon were recombined into appropriate binary destination vectors using a variety of pSITE or pSITE II vectors (Chakrabarty et al., 2007; Goodin et al., 2007; Martin et al., 2009). Protein localization vectors used in this study were pSITE-2CA [green fluorescent protein (GFP) fusions] and pSITEII-6C1 (TagRFP fusions) (Fig. 1). For BiFC assays, all INSV proteins were tested as fusions to the amino- and carboxy-terminal portions of yellow fluorescent protein in all pairwise combinations [pSITE-BiFC-nEYFP-C1 and pSITE-BiFC-cEYFP-C1 (Fig. 1); Bandyopadhyay et al., 2010]. INSV Gn and Gc were also cloned into BiFC-N1 vectors (Fig. 1; Martin et al., 2009) and tested in all combinations and orientations. Glutathione-S-transferase (GST) was used as non-binding control. Recombinant vectors were transformed into Agrobacterium tumefaciens LBA4404 and agroinfiltrations were done as described by Goodin et al. (2005). Each expression construct was examined by laser scanning confocal microscopy (Goodin et al., 2005) in sections taken from at least two leaves from each of three separate plants in three independent experiments. Several hundred cells were examined for each experiment and at least three high-resolution micrographs were acquired for each construct.

When expressed as a GFP fusion, the INSV N protein formed many different sized aggregates in the cell periphery of wild-type and red fluorescent protein (RFP)–H2B nuclear marker plants (Fig. 2a, i–iii), indicating a potential N protein self-interaction. Some of the aggregates were located close to but outside of the cell nuclei. Similar cytoplasmic aggregates were detected previously when N- and C-terminal fusions of GFP with TSWV N protein were expressed in N. benthamiana leaves (Lacorte et al., 2007). Fusions of INSV N protein with RFP formed similar aggregates in the cell periphery that co-localized with endoplasmic reticulum (ER) targeted GFP in 16c transgenic plants (data not shown). Such a co-localization was not observed for TSWV N protein in tobacco protoplasts or in BHK21 cells (Ribeiro et al., 2009; Snippe et al., 2005) and may only be detectable by live plant cell imaging of intact leaves or may depend on cytoplasmic protein accumulation and aggregation levels.

GFP fusions of the non-structural proteins NSm and NSs also specifically localized to the cell periphery in wild-type and RFP–H2B transgenic plants. NSs showed a strong punctate distribution when fused to GFP (Fig. 2a, iv–vi), which was less pronounced when fused to RFP (data not shown). NSm on the other hand appeared smoothly distributed and relatively strongly expressed compared with the other viral proteins (Fig. 2a, vii–ix). Both proteins co-localized with GFP–ER when expressed as RFP fusions (data not shown). The smooth distribution of NSm on the cell periphery shows localization along the entire ER not specifically the plasmodesmata as observed for TSWV (Kormelink et al., 2011), which may indicate additional functions or host interactions.

GFP fusions of the mature glycoproteins, Gn and Gc, localized to the cell periphery and co-localized with ER in RFP–ER transgenic plants (Fig. 2b, i–iii and iv–vi). GFP–Gn localized exclusively to the cell periphery (Fig. 2a, x–xii), whereas, GFP–Gc localized to both the cell periphery and the nucleus (Fig. 2a, xiii–xv; Fig. 2b, iv–vi).

When the five viral proteins were tested by BiFC in all pairwise combinations, three interactions were detected: N protein homotypic interaction (Fig. 3a–c), NSm protein homotypic interaction (Fig. 3d–f) and NSm–N protein heterotypic interaction (Fig. 3g–i). The N protein self-interaction resulted in differently sized aggregates localized to the cell periphery (Fig. 3a–c) similar to those seen with GFP- or RFP-tagged N protein (Fig. 2a, a–c). The NSm self-interaction had a smooth cell periphery distribution (Fig. 3d–f), similar to GFP-tagged NSm protein (Fig. 2a, vii–ix). The heterotypic interaction had a smooth cell periphery distribution with some punctate spots (Fig. 3g–i) and was only detected when INSV NSm was fused to the amino-terminal portion of YFP and INSV N was fused to the carboxy-terminal portion of YFP. The strong self-interaction of N protein molecules also led to the formation of some aggregates in the interaction with NSm, indicating potential multi-molecule complexes. No interactions involving the NSs or either glycoprotein were detected by BiFC.

Tospoviruses replicate in the cytoplasm of infected cells (Kormelink et al., 2011). All INSV proteins analysed in this study localized to the cell periphery confirming cytoplasmic replication. Research on tospovirus protein function and protein–protein interactions has largely been limited to TSWV in diverse systems such as animal, plant and yeast cells and using a variety of interaction assays. This report on INSV protein localization and interactions is the first for tospovirus N, NSs, NSm, Gn and Gc proteins in the live plant cell system. The infectious ribonucleoprotein core of tospoviruses consists of genomic RNA tightly associated with many molecules of N protein and small amounts of the viral RdRP (Kormelink et al., 2011). The observed INSV N protein homotypic interaction is consistent with this essential association. TSWV N protein self-interactions
Fig. 2. Confocal micrographs of the localization of INSV proteins in relation to (a) the red nuclear marker histone 2B (RFP–H2B) or (b) red endoplasmic reticulum marker (RFP–ER) in transgenic *N. benthamiana* epidermal leaf cells. Images represent INSV fusion proteins to the C terminus of GFP (see Fig. 1). (a) From left to right, the first column shows GFP-gene fusion, the second RFP–H2B, and the last column shows the overlay of the two images. (i–iii) Co-expression of GFP–INSV N with RFP–H2B. (iv–vi) Co-expression of GFP–INSV NSs with RFP–H2B. (vii–ix) Co-expression of GFP–INSV NSm with RFP–H2B. (x–xii) Co-expression of GFP–INSV Gn with RFP–H2B. (xiii–xv) Co-expression of GFP–INSV Gc with RFP–H2B. (b) From left to right, the first column shows GFP-gene fusion, the second RFP–ER, and the last column shows the overlay of the two images. (i–iii) Co-expression of GFP–INSV Gn with RFP–ER. (iv–vi) Co-expression of GFP–INSV Gc with RFP–ER. Regions of co-localization appear in orange-yellow in the overlay. Micrographs shown are representative of at least 50 cells examined. Bars, 10 μm.
and multimerization were first detected by yeast two-hybrid analysis and two interacting domains were identified and characterized (Uhrig et al., 1999). Subsequently, TSWV N protein homotypic interactions were visualized by fluorescence microscopy in baby hamster kidney cells (Snippe et al., 2005). Recently, Zilian & Maiss (2011) demonstrated an in planta N protein homotypic interaction for Capsicum chlorosis tospovirus by using BiFC.

The tospovirus glycoproteins are required for transmissibility by thrips (Sin et al., 2005). In planta TSWV Gn and Gc induce membrane deformation and interact with each other and with the N protein to facilitate particle assembly at the Golgi complex (Snippe et al., 2007; Ribeiro et al., 2009). Localization and membrane interactions of transiently expressed TSWV Gn and Gc protein YFP fusions were studied in tobacco protoplasts (Ribeiro et al., 2008). When expressed separately, TSWV YFP-Gn was found in the ER and Golgi membranes and YFP-Gc solely in the ER. INSV GFP-Gn was found in the ER membranes and in punctate ER-associated bodies, but did not co-localize with a reference Golgi marker protein (data not shown); INSV GFP-Gc also localized solely to the ER when expressed in epidermal leaf cells. Interestingly, INSV GFP-Gc also localized to the nucleus, a glycoprotein location not previously reported for any other tospovirus. The function of Gc in the nucleus is unknown but may involve interactions with nuclear membranes, host proteins or nucleic acids to redirect cellular processes. Although, we could express and localize the INSV Gn and Gc proteins as

![Fig. 3. Confocal micrographs showing INSV protein interactions determined by BiFC. Interaction assays were done in leaf epidermal cells of transgenic N. benthamiana expressing cyan fluorescent protein fused to the nuclear marker histone 2B (CFP–H2B). Shown are the localization of CFP–H2B (nucleus, column 1), interaction assay (BiFC, column 2) and a merge of the two preceding panels (overlay, column 3). Proteins listed first in the pair of interactors were expressed as C-terminal fusions to the amino-terminal half of YFP. Those listed second were expressed as C-terminal fusions to the carboxy-terminal half of YFP. All protein fusions to each half of YFP were tested in all pairwise combinations, of which a subset of detectable interactions is shown here: (a–c) N/N, (d–f) NSm/NSm, (g–i) NSm/N. Representative results using GST fusions as non-binding controls with the INSV interactors are shown in (j–l) GST/N and (m–o) GST/NSm. Micrographs shown are representative of at least 50 cells examined. Bars, 10 µm.](http://vir.sgmjournals.org)
fusions with GFP and RFP, we did not detect any homo- or heterotypic interactions involving these viral glycoproteins using BiFC. This may have been due to low-level protein expression or steric constraints preventing the YFP halves from coming together. In our BiFC assays of the INSV glycoproteins, YFP halves were attached to the amino- or carboxy-termini of the viral proteins and tested in all combinations, but no interaction was detected. Such interactions may be detectable in alternative protein interaction assays such as yeast-two hybrid or pull-down. Alternatively, some interactions may only occur in the presence of replicating virus, requiring more than one interaction partner.

TSWV NSm functions as a cell-to-cell movement protein that associates with ribonucleoprotein (RNP) complexes and localizes in tubular structures at plasmodesmata (Kormelink et al., 2011). In this study, INSV NSm protein interacted with the N protein and with itself in BiFC, as would be expected for functions in RNP binding and tubule formation. Interaction between NSm and N proteins (and viral RNA) likely provides a molecular basis for specific recognition of nucleocapsid structures and for intra- and intercellular trafficking by interactions with different host proteins (Soellick et al., 2000; Paape et al., 2006; Kormelink et al., 2011).

Tospovirus NSs protein interferes in plant RNA silencing defence by sequestering small RNAs to prevent uploading into RNA-induced silencing complexes (Kormelink et al., 2011) and interacts with several host RNA silencing pathway components. However, no interactions with other viral proteins have been reported. We also did not detect any homotypic or heterotypic viral protein interactions involving the INSV NSs protein by BiFC.

This communication shows for the first time the localization and interactions of transiently expressed INSV structural and non-structural proteins, except for RdRP, in live plant cells and relates these data to known functions of viral proteins of the type member of the tospoviruses, TSWV. Future studies of INSV proteins will address the entry and putative function of the Gc protein in the nucleus, use of alternative protein interaction assays to identify expected, putative function of the Gc protein in the nucleus, use of fluorescence microscopy to visualise homotypic interactions of viral proteins and tested in all combinations, but no interaction was detected. Such interactions may be detectable in alternative protein interaction assays such as yeast-two hybrid or pull-down. Alternatively, some interactions may only occur in the presence of replicating virus, requiring more than one interaction partner.

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