A Host-Factor Interaction and Localization Map for a Plant-Adapted Rhabdovirus Implicates Cytoplasm-Tethered Transcription Activators in Cell-to-Cell Movement

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To identify host factors that play critical roles in processes, including cell-to-cell movement of plant-adapted rhabdoviruses, we constructed and validated a high-resolution *Nicotiana benthamiana* yeast two-hybrid library. The library was screened with the putative movement protein (sc4), nucleocapsid (N), and matrix (M) proteins of *Sonchus yellow net virus* (SYNV). This resulted in identification of 31 potential host factors. Steady-state localization studies using autofluorescent protein fusions to full-length clones of interactors were conducted in transgenic *N. benthamiana* marker lines. Bimolecular fluorescence complementation assays were used to validate two-hybrid interactions. The sc4 interactor, sc4i21, localized to microtubules. The N interactor, Ni67, localized to punctuate loci on the endoplasmic reticulum. These two proteins are 84% identical homologues of the *Arabidopsis* phloem-associated transcription activator AtVOZ1, and contain functional nuclear localization signals. Sc4i17 is a microtubule-associated motor protein. The M interactor, Mi7, is a nuclear-localized transcription factor. Combined with a binary interaction map for SYNV proteins, our data support a model in which the SYNV nucleocapsids are exported from the nucleus and moved cell-to-cell by transcription activators tethered in the cytoplasm.

In order to infect systemically, plant viruses recruit host factors that facilitate cell-to-cell and long-distance movement (Chen and Citovsky 2003; Tzfira and Citovsky 2008; Epel 2009; Harries et al. 2009; Wang et al. 2009; Lewis and Lazarowitz 2010). Despite the wealth of information for the movement of many genetically diverse plant viruses, there is a paucity of such information for the plant-infecting rhabdoviruses.

The nucleorhabdoviruses are those plant-adapted rhabdoviruses that replicate in nuclei of infected plant cells (Jackson et al. 2005; Tordo et al. 2005). These viruses share many of the structural features of animal rhabdoviruses, such as *Vesicular stomatitis virus* (VSV) (Jackson et al. 2005). As such, their minus-sense single-stranded RNA genome is not infectious. Instead, the minimal infectious unit of these viruses is a nucleocapsid composed of the genomic RNA encapsidated over its entire length by a nucleocapsid (N) protein, and associated with this complex are the phospho (P) and polymerase (L) pro-

teins. During morphogenesis, the nucleocapsid is condensed by the matrix (M) protein to form viral “cores” that bud through the inner nuclear membrane into the perinuclear space, acquiring a host-derived lipid envelope and viral-encoded glycoprotein (G) in the process (van Beek et al. 1985). Currently, molecular details of how plant-adapted rhabdoviruses move from sites of replication and morphogenesis into adjacent cells are lacking. Two possible models for cell-to-cell movement of these viruses are considered here. First, mature virions may bud from the perinuclear space through the outer nuclear membrane, similarly to a model proposed for release of herpes virus particles (Farnsworth et al. 2007; Mettenleiter et al. 2009; Sagou et al. 2010). Alternatively, the nucleocapsid may be exported from the nucleus, as occurs with influenza and plant DNA viruses (Sanderfoot et al. 1996; O’Neill et al. 1998; Boulo et al. 2007). Whichever model is correct, it has long been suspected that the sc4 protein of *Sonchus yellow net virus* (SYNV) facilitates cell-to-cell movement (Scholthof et al. 1994; Melcher 2000; Goodin et al. 2001; Huang et al. 2005). Also implicated in the formation of SYNV movement complexes is the M protein, which has been shown to form mobile complexes associated with the endoplasmic reticulum (ER) in virus-infected cells (Goodin et al. 2007). Therefore, using SYNV proteins as baits, screens of a high-resolution *Nicotiana benthamiana* yeast-two hybrid library and live-cell imaging were performed. We provide evidence that the cell-to-cell movement of plant-adapted rhabdoviruses requires cytoplasm-tethered transcription activators that facilitate formation and transport of an ER- and microtubule-associated complex.

RESULTS

Construction of an interaction matrix for SYNV proteins.

Bimolecular fluorescence complementation (BiFC) was used to determine the binary interactions and localization patterns of SYNV protein complexes. We chose to use BiFC given that it provided simultaneous interaction and localization data in planta (Citovsky et al. 2006; Martin et al. 2009; Bandyopadhyay et al. 2010). The SYNV N, P, sc4, M, and G proteins were tested in all pairwise interactions and against glutathione-S-transferase (GST), which served as a nonbinding control (Fig. 1). The L protein was not included in these studies due to the inability to express this 240-kDa protein. We did not observe binding between any of the SYNV proteins and GST. Therefore, we provide only one example of this control

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experiment (Fig. 1A1 to A3). The sc4–sc4 interaction was detected exclusively on the periphery of cells (Fig. 1B1 to B3). In contrast, the sc4–G interaction was detected on both the cell periphery and nuclear membranes (Fig. 1C1 to C3). The G–G interaction was detected primarily on perinuclear membranes (Fig. 1D1 to D3). The M protein self-interaction was detected in subnuclear loci (Fig. 1E1 to E3), whereas the N–N interaction was dispersed throughout the nucleus but excluded from the nucleolus (Fig. 1F1 to F3). As reported previously (Goodin et al. 2001; Martin et al. 2009), the N–P interaction was subnuclear (Fig. 1G1 to G3), while that of P–P was nuclear and cytoplasmic (Fig. 1H1 to H3). All other pairwise combinations of SYN protein interactions were negative in BiFC assays.

Characteristics of host factors that interact with SYN proteins.

We used high-resolution screens of an *N. benthamiana* yeast two-hybrid library to identify proteins that interact with the N, M, and sc4 proteins. The high resolution of this library refers to the number of independent cDNA clones that compose the library. For this study, a library containing 10 million independent clones was constructed. This contrasts to a previously reported *N. benthamiana* yeast two-hybrid library composed of 1.2 million clones (Jiménez et al. 2006). The greater the number of independent clones, the better the possibility of finding rare or weak-interacting host factors.

Following protocols reported previously (Formstecher et al. 2005), as well as bioinformatic characterization, the interactors were assigned confidence scores of A (very high confidence), B (high confidence), C (good confidence), and D (moderate confidence, likely to include false positives). Low-confidence interactors were removed from the dataset. Additionally, interactors that did not have significant matches in protein sequence databases were also excluded. In total, 31 unique interactors were given priority for further study: 8 N protein interactors, 10 sc4 interactors, and 16 M protein interactors (data not shown). Five interactors, including sc4i17, were common to the M and sc4 screens. Four of the very highest confidence interactors (sc4i17, sc4i21, Mi7, and Ni67) were selected for this study.

Using a variety of web-based proteomics tools, we characterized the selected SYN host factors selected for this study (Table 1). The deduced amino acid sequence of the M interactor, Mi7, encodes a small 21-kDa basic protein with an isoelectric point (pI) value of 9.7. Mi7 contains a DUF640 domain conserved in such transcription factors, including the

Arabidopsis light-sensitive hypocotyl 10 protein (TAIR reference AT1G78815; GenBank accession number NM_106529). Mi7 was predicted to contain both a PYKKKKK nuclear localization signal (NLS) starting at amino acid 155 and a VLEFL RYLD nuclear export signal (NES) starting at amino acid residue 66. The steady-state localization pattern for Mi7, shown below, was nuclear.

The sc4i17 protein was predicted to be an 80-kDa protein with a pI of 6.0. This protein is 98% identical to the *N. tabacum* BY-2 kinesin-like protein 10 (Matsui et al. 2001). Consistent with the predicted motor domain at amino acids 186 to 508 in the deduced protein sequence, sc4i17 localized to punctate loci on microtubules in *N. benthamiana* leaf epidermal cells.

Fig. 1. Single-section confocal micrographs showing *Sonchus yellow net virus* (SYNV) protein interactions determined by bimolecular fluorescence complementation (BiFC). Interaction assays were conducted in leaf epidermal cells of transgenic *Nicotiana 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 merge of the two preceding panels (overlay, column 3). Proteins listed first in the pair of interactors were expressed as fusions to the amino-terminal half of yellow fluorescent protein (YFP). Those listed second were expressed as fusions to the carboxy-terminal half of YFP. However, protein fusions to each half of YFP were tested in all pairwise interactions, of which a subset is shown here. **A1 to A3**, Only one representative of the results obtained using glutathione-S-transferase (GST) as a nonbinding control is provided here. However, no SYN protein shown here interacted with GST. All pairwise interactions for SYN proteins, excluding L, were tested. BiFC-positive interactions were observed for **B1 to B3**, sc4/sc4; **C1 to C3**, G/sc4; **D1 to D3**, G/G; **E1 to E3**, M/M; **F1 to F3**, N/N; **G1 to G3**, N/P; and **H1 to H3**, P/P. The following pairwise combinations were BiFC negative: N/M, N/sc4, N/G, P/M, P/sc4, P/G, M/sc4, and M/G.

The sc4 interactor, sc4i21, is a 53-kDa protein, pI 5.8, that contains a conserved “no apical meristem” (NAM) domain found in some transcription factors, such as the phloem-associated *Arabidopsis* vascular one–zinc finger protein 1 (AtVOZ1) (Mitsuda et al. 2004), with which it shares 65% identity (E value = 3e-165). Although sc4i21 contains a predicted NLS

(314-KPRR-317) and NES (382-LYRLELKLVD-391), its steady state localization was on microtubules. Interestingly, Ni67, a 53-kDa protein, pI 5.5, shares 84% sequence identity with sc4i21, with identically positioned NLS and NES sequences. However, the steady-state localization of Ni67 was located in the ER, as shown below.

Table 1. *Sonchus yellow net virus* (SYNV) host factors characterized in this study^a

Interactor	MW/pI	Domain	Homologue	NLS	NES	Localization
Mi7	21/9.7	DUF640/TF	AtLSH10	+	+	Nucleus
Sc4i17	80/6.0	Motor	MT-kinesin	–	–	Microtubule
Sc4i21	53/5.8	NAM/TF	AtVOZ1	+	+	Microtubule
Ni67	53/5.5	NAM/TF	AtVOZ1	+	+	Endoplasmic reticulum

^a Features of these proteins were determined by predictive algorithms. Molecular weight (MW) in kilodaltons and isoelectric point (pI) of each protein were predicted from the deduced amino acid sequence for each gene. Domain searches identified conserved domains in transcription factors (TF) or motor proteins. Protein with the highest named homologue in BLAST searches included the *Arabidopsis* light-sensitive hypocotyl 10 (AtLSH10), a *Nicotiana benthamiana* BY-2 microtubule-associated motor kinesin (MT-kinesin), and the *Arabidopsis* vascular one-zinc-finger 1 (AtVOZ1) proteins. The presence or absence of nuclear localization signal (NLS) and nuclear export signal (NES) sequences are also shown, as is the predominant site of localization of these proteins in steady-state observations.

Fig. 2. A, Single-plane confocal micrographs showing the relationship between the localization patterns of A1, green fluorescent protein–endoplasmic reticulum (GFP-ER) and A2, sc4i21. A3, Merger of A1 and A2. B, Z-stacks of confocal micrographs to show the relationship between B1, GFP-labeled actin filaments in leaf epidermal cells of transgenic *N. benthamiana* plants expressing GFP-Talin and B2, sc4i21 expressed by agroinfiltration. B3, Overlay of B1 and B2. B4, Under conditions that completely disrupt actin filaments (10 μM LatB), B5, localization of sc4i21 was unaffected. B6, Overlay of B4 and B5. C1 and C4, GFP-labeled microtubules colocalize with C2, sc4i21 and C5, sc4i17. C3 and C6, Overlay of these images. D, Confocal micrographs to further establish the microtubule association of sc4 interactors. D1, Under control conditions, RFP:sc4i21 appeared as filaments. D2, Following treatment with 100 μM oryzalin, RFP:sc4i21 was detected as disorganized aggregates. D3, In single-plane confocal micrographs taken at mid-cell, RFP:sc4i17 was easily detected continuously around the cell periphery. D4, Following treatment with oryzalin, RFP:sc4i17 is detected primarily in discrete puncta on the cell periphery.

Microtubule association of sc4i17 and sc4i21.

To validate the protein prediction data, we expressed full-length open reading frames (ORF) for sc4i17 and sc4i21 as tag-red fluorescent protein (tRFP) fusions in fluorescent marker lines of *N. benthamiana* (Goodin et al. 2007; Martin et al. 2009). Sc4i21 localized to filaments that only partially colocalized with the ER (Fig. 2A1 to A3). To establish the nature of the sc4i21 filaments, we expressed tRFP-tagged sc4i21 in transgenic *N. benthamiana* plants expressing green fluorescent protein (GFP):talin to label actin filaments (Fig. 2B1 to B3). Treatment of this tissue with 10 mM Latrunculin B resulted in complete disruption of actin filaments, whereas there was little

effect on localization of sc4i21 (Fig. 2B4 to B6). Coexpression of tRFP-tagged sc4i21 (Fig. 2C1 to C3) or sc4i17 (Fig. 2C4 to C6) with a GFP-microtubule-binding domain fusion (GFP:MBD) (Marc et al. 1998) suggested that both tRFP fusions colocalize with microtubules. To provide further support for microtubule association, we treated leaves expressing tRFP-sc4i21 or tRFP-sc4i17 with 100 mM oryzalin. Under drug treatment, sc4i21 filaments were completely disrupted (Fig. 2D1 and D2), while sc4i17, which was most easily viewed at the cell periphery, was detected only at punctate loci on cell walls following oryzalin treatment (Fig. 2D3 and D4).

Homologous and heterologous interactions between host factors and SYNv proteins.

Given that transcription factors often function as dimers or in higher-order oligomers (Amoutzias et al. 2008), we examined the ability of Mi7, Ni67, sc4i17, and sc4i21 to associate with each other as well as with SYNv proteins. In the sections below, we provide micrographs for the results of BiFC experiments conducted with host factors and SYNv proteins. It was found that their interactions with different partners could dramatically alter the localization of some host factors. The results of these assays are summarized in Table 2. Note that Ni67

Table 2. Subcellular loci at which binary complexes of host factors and the nucleocapsid (N), matrix (M), and sc4 proteins were found^a

Loci	N	M	sc4	Ni67	Mi7	sc4i17	sc4i21
Ni67	Nu	–	–	ER	–	–	Per
Mi7	–	Nu	–	–	Nu	–	–
sc4i17	–	Nu	MT	–	–	MT	MT
sc4i21	–	–	Per	Per	–	MT	Per/MT

^aNu = nucleus, ER = endoplasmic reticulum, Per = periphery, and MT = microtubule; – indicates that no interaction was detected.

Fig. 3. Single-plane confocal micrographs showing the relationship between the localization patterns of **A1**, green fluorescent protein–endoplasmic reticulum (GFP-ER) and **A2**, Ni67 and **A3**, the merger of these two panels (overlay). **B**, Region (green box) selected in **A1** shows that **B1**, ER rings encircle **B2**, Ni67 complexes. **B3**, Merger of **B1** and **B2**. Despite its steady state localization in the cytoplasm, use of a nuclear marker line of **C1**, *Nicotiana benthamiana* shows that the **C2**, interaction of Ni67 and *Sonchus yellow net virus* N protein was nuclear. **C3**, Merger of **C1** and **C2**. Protein localization was conducted in leaf epidermal cells of transgenic *N. benthamiana* cells expressing GFP targeted to the ER (GFP-ER) or cyan fluorescent protein targeted to the nucleus (CFP:H2B).

was detected within nuclei, on ER membranes, or at the cell periphery depending on its interacting partner. Similarly, sc4i17 was found in the nucleus or on microtubules when interacting with M or sc4, respectively.

Localization and interaction of Ni67.

We expressed tRFP-tagged Ni67 in transgenic *N. benthamiana* plants expressing GFP-ER. Under steady-state observations, Ni67 accumulated in punctate loci on ER (Fig. 3A1 to A3). Examination of these puncta revealed Ni67 complexes surrounded by ER-derived rings (Fig. 3B1 to B3). In contrast to its steady-state localization pattern, BiFC established that Ni67 interacts with the N protein in nuclei (Fig. 3C1 to C3).

Localization and interaction of Mi7.

Mi7 localized exclusively to nuclei when expressed as tRFP fusions in GFP-ER transgenic plants (Fig. 4A to C). BiFC validated the yeast two-hybrid results given that M interacted with Mi7 in nuclei (Fig. 4D to F). Additionally, we show that Mi7 interacted with itself (Fig. 4G to I) but not with GST (Fig. 4J to L).

Binary complexes

between sc4, M, and host factors sc4i17 and sc4i21.

The host factor sc4i17 was detected in two-hybrid screens using either sc4 or M as bait. Therefore, we determined whether interaction with different viral proteins could change the locali-

Fig. 4. Single-plane confocal micrographs showing the relationship between the localization patterns of A, Mi7 in B, green fluorescent protein–endoplasmic reticulum plants (GFP-ER). C, Merger of A and B. D, G, and J, Bimolecular fluorescence complementation assays conducted in leaf epidermal cells of transgenic *N. benthamiana* plants expressing a nuclear marker protein show that E, the Mi7/M interaction was F, nuclear. H, The Mi7/Mi7 interaction was also I, nuclear. K and L, Control experiments showed that Mi7 did not interact with the nonbinding control glutathione-S-transferase.

zation pattern of sc4i17. Interaction between M and sc4i17 was detected as mobile complexes within nuclei (Fig. 5A to C). The sc4/sc4i21 interaction was detected on the periphery of cells (Fig. 5D to F). In contrast to the M–sc4i17 interaction, the sc4–sc4i17 was detected on filamentous complexes in the cytoplasm and around nuclei (Fig. 5G and H).

Binary complexes between host factors.

In addition to interactions between SYN V proteins and host factors, we tested the ability of the host factors to form binary complexes. Sc4i17/sc4i17 complexes formed on filamentous structures that were dispersed throughout cells (Fig. 6A1 to A3). Similar to its steady-state localization pattern using tRFP fusions, Ni67/Ni67 complexes accumulated in a reticulate pattern in the cytoplasm (Fig. 6B1 to B3). The sc4i21/Ni67 complexes were detected almost exclusively on punctate loci on cell periphery (Fig. 6C1 to C3). The sc4i21–sc4i21 interaction was detected on cell periphery (Fig. 6D1 to D3) as well as on cortical filamentous complexes (Fig. 6D4).

Mi7, sc4i21, and Ni67 contain functional NLS sequences.

The predicted NES and NLS sequences in Sc4i17 and Ni67 and the intranuclear Ni67–N interaction suggested that, despite their cytoplasmic accumulation at steady state, these proteins are capable of nuclear import. Therefore, Mi7, sc4i17, sc4i21, and Ni67 were screened in a yeast-based nuclear import assay (NIA) (Fig. 7). In this assay, only proteins containing a functional NLS will facilitate the nuclear import of a transcriptional activator required for expression of a reporter gene in yeast cells (Zaltsman et al. 2007; Bandyopadhyay et al. 2010). Entirely consistent with computational predictions, Mi7, sc4i21, and Ni67 were all NIA positive, as was the control protein *N. benthamiana* histone 2B. The sc4i17 protein and the control using maltose-binding protein (MBP) were NIA-negative.

Multiple forms of SYN V-G accumulate in plant cells.

Interpretation of the data showing the interaction of sc4 and G in BiFC assays presented a topological problem in that the amino terminus of G should be ER luminal and, thus, inaccessible to sc4, which lacks a signal peptide. However, it is known that several negative-strand RNA viruses produce truncated

and soluble forms of their G proteins (Graeve et al. 1986; Volchkov et al. 1998; Teng et al. 2001). We postulated that SYN V may do the same and that it is, in fact, a truncated form of G that interacts with sc4. Indeed, Western immunoblotting showed that two stable forms of G accumulated in leaf tissues in the context of both viral infections and agroinfiltration-based expression of a recombinant G protein (Fig. 8A and B). The mature form of G migrated at 70 kDa on denaturing polyacrylamide gels, while the truncated form, hereafter called G*, has an estimated molecular weight of 51 kDa.

To determine the ability of G* to interact with sc4, we utilized a 51-kDa amino terminal portion of G that lacked its transmembrane domain and carboxy terminus in BiFC experiments. G* was detected in binary complexes located primarily in perinuclear ER (Fig. 8C1 to C3). G* retained the ability to interact with full-length G, and binary complexes of these proteins were found on nuclear and endomembranes (Fig. 8D1 to D3). G*/sc4 complexes were found on the nuclear envelope and on punctate loci along the cell periphery (Fig. 8E1 to E4). G* did not form complexes with GST (Fig. 8F1 to F3). Although the precise carboxy terminus of the native G* has not been mapped, these results were reproducible with both 51- and 53-kDa amino-terminal portions of G (data not shown).

A host factor protein interaction map for SYN V.

The protein localization and interaction data presented above were integrated into a comprehensive map (Fig. 9). All nine proteins used in this study were capable of self-interactions. Two host factors, sc4i21 and Ni67, formed heterologous interactions with each other that resulted in a change in their steady-state localization patterns. One host factor, sc4i17, interacted with both sc4 and M. In contrast to *Potato yellow dwarf virus* (PYDV) (Bandyopadhyay et al. in press), SYN V proteins formed few heterologous associations (N/P and sc4/G).

DISCUSSION

Protein interaction maps, particularly when supported by protein localization data, are critical for understanding molecular mechanisms that underlie viral infection and transmission processes (Uetz et al. 2005; Guo et al. 2008; Shapira et al.

Fig. 5. Single-plane confocal micrographs of bimolecular fluorescence complementation assays conducted in leaf epidermal cells of transgenic *Nicotiana benthamiana* plants expressing a nuclear marker protein (CFP:H2B). **A to C**, The M/sc4i17 interaction resulted in the formation of **B**, mobile complexes that are **C**, nuclear localized. **D through F**, The sc4/sc4i21 interaction was detected most strongly **E**, on the cell periphery. **G**, The sc4/sc4i17 was detected on filamentous complexes that radiate from the **H**, nucleus (inset).

2009; Bandyopadhyay, et al. 2010). Moreover, they permit a rapid means to infer different mechanisms by which related viruses might conduct particular processes. For example, a comparison of protein interaction data for the nucleorhabdoviruses suggests that at least two different strategies for cell-to-cell movement may be employed by *Rice yellow stunt virus* (RYSV), PYDV, and SYN, the movement proteins for which are referred to as P3, Y, and sc4, respectively. In the case of RYSV, the P3 protein is thought to bind directly to nucleocapsids. In contrast, no comparable Y–N or sc4–N interactions have been detected (Bandyopadhyay, et al. 2010; this study). For PYDV, Y interacts with the matrix and glyco proteins, which may permit the formation of a movement complex similar to that proposed here for SYN. For both PYDV and SYN, interactions with either M or G proteins result in relocalization of the movement proteins from the cell periphery to the nucleus, the site of viral replication and morphogenesis. Overall, the PYDV and SYN protein interaction maps are

similar with all proteins, except the PYDV phosphoprotein, capable of forming at least binary associations (Bandyopadhyay et al. 2010).

In order to gain an in-depth understanding of viral infection processes, viral protein interaction maps must be expanded to include host proteins (Uetz et al. 2005; Shapira et al. 2009). Although it is generally established that, in order to move from initially infected cells into adjacent cells, plant viruses employ the ER (Bamunusinghe et al. 2009) and at least one type of protein filament, be it actin (Prokhnovsky et al. 2005) or microtubules (Epel 2009; Sambade and Heinlein 2009), no additional host factors have been implicated in the movement of plant-adapted rhabdoviruses.

Under the assumption that binding to the N protein is the equivalent of binding to the nucleocapsid, we propose a model for SYN movement that requires the N, M, sc4, and G or G* proteins of SYN and the host factors sc4i17, sc4i21, Mi7, and Ni67 (Fig. 10). We believe this assumption, also made by Huang

Fig. 6. Single plane confocal micrographs of bimolecular fluorescence complementation (BiFC) assays conducted in leaf epidermal cells of transgenic *Nicotiana benthamiana* plants expressing a nuclear marker protein. Panels show the location of nuclei (CFP-H2B) and protein interaction assay (BiFC) and mergers of these two images (overlay). **A1** to **A3**, Sc4i17/sc4i17 form filamentous complexes. **B1** to **B3**, Ni67/Ni67 complexes were dispersed throughout the cytoplasm. **C1** to **C3**, Sc4i21/Ni67 complexes accumulated in punctate loci on the cell periphery. Sc4i21/sc4i21 complexes accumulated in punctate loci on the cell periphery in **D2** and **D3**, mid-cell micrographs and **D4**, on filaments when viewed at the top or bottom of cells. None of these host factors interacted with the nonbinding control glutathione-S-transferase (data not shown).

and associates (2005), to be valid, given that the N protein readily forms viroplasm-like complexes even in the absence of other viral proteins (Martins et al. 1998; Deng et al. 2007) and that N proteins can self-assemble into ribonucleoprotein complexes in the absence of viral RNA (Green et al. 2000).

By virtue of their much larger molecular weights, it is clear that Ni67 and sc4i21 are distinct from the 10-kDa MBF1 transcriptional coactivator that was shown to interact with p30 of *Tobacco mosaic virus* (TMV) (Matsushita et al. 2002). Interestingly, both sc4i21 and Ni67 contain a NAM domain, as does the smaller 32-kDa ATAF2 that binds to the helicase domain of the TMV replicase and that is involved in suppression of systemic host defense (Wang et al. 2009). Thus, to our knowledge, neither the host factors reported here nor their homologues in other plants have been shown to participate in the biology of other plant viruses.

We propose that nucleocapsids that are not condensed and budded into the perinuclear space associated with Ni67 and Mi7, both of which contain functional nuclear import and predicted export signals. The use of two factors for export is attractive because it could provide a regulatory mechanism for exporting only nucleocapsids, or partially condensed cores, and not simply oligomers of the N and M proteins. We find it intriguing that VOZ1, the closest named homologue of both sc4i21 and Ni67, is expressed primarily in the phloem (Mitsuda et al. 2004). It stands to reason that a nucleocapsid destined for cell-to-cell movement would associate with phloem-associated proteins, particularly if it was a non-cell autonomous protein (NCAP) with intrinsic ability to move between cells (Oparka 2004). Once exported, Ni67 may anchor nucleocapsids onto ER membranes, bringing with it the associated M and Mi7 proteins, both of which associate with mobile ER-associated complexes (Goodin et al. 2007) (data not shown). The ER-associated complex could then be tethered to microtubules via a complex containing the motor-kinesin, sc4i17, as well as sc4i21 and G*. Once formed, the complete ER-associ-

ated movement complex could track on microtubules toward plasmodesmata in a manner similar to that of NCAP. Given the punctate localization of the Ni67–sc4i21 on cell periphery, which could be plasmodesmata, we propose that, upon reach-

Fig. 7. Yeast-based assay for identification of proteins containing a functional nuclear localization signal (NLS). Positive- (H2B) and negative-control (MBP) proteins or *Nicotiana benthamiana* proteins (Mi7, sc4i17, sc4i21, and Ni67) were expressed from pNIA-DEST in yeast strain L40. Top panel: yeast dropout media supplemented with histidine. Lower panel: yeast dropout media lacking histidine. Only those proteins containing a predicted NLS (Mi7, sc4i21, and Ni67) were able to facilitate yeast growth on media lacking histidine.

Fig. 8. Two forms of the *Sonchus yellow net virus* (SYNV) glycoprotein accumulate in **A**, virus-infected cells (SYNV) or **B**, leaf-epidermal cells of *Nicotiana benthamiana*, in which a recombinant G protein was expressed by agroinfiltration (^{FLAG}G). The full-length G protein (G) is approximately 70 kDa whereas the truncated form, G*, is approximately 50 kDa. A truncated G protein lacking its transmembrane, and carboxy-terminus interacted with **C1** to **C3**, itself; **D1** to **D3**, full-length G; and **E1** to **E3**, sc4 but not **F1** to **F3**, glutathione-S-transferase. The boxed area in **E3** is enlarged in **E4** to show that the G*–sc4 interaction was detected primarily on punctate loci on the cell periphery. Bimolecular fluorescence complementation (BiFC) conducted in leaf epidermal cells of transgenic *N. benthamiana* plants expressing a fluorescent nuclear marker (CFP:H2B) was used to determine protein interactions.

ing this structure, a Ni67–sc4i21 interaction could provide a mechanism to release the nucleocapsid into the adjacent cell. Some such release of the nucleocapsid from the movement complex is required in order to allow the nucleocapsid to be ferried to the nucleus, probably via an importin- α -mediated process (Deng et al. 2007), to initiate the next infection cycle (Jackson et al. 2005). Unfortunately, the requirement for these host factors in cell-to-cell movement could not be supported by RNA-silencing experiments (Park et al. 2009), due to the inability of virus-induced gene silencing (Liu et al. 2002) to significantly reduce the steady-state levels of host factor mRNAs, which accumulated to very low levels in both mock-inoculated and SYN-1 infected plants (data not shown). A greater degree of silencing, probably in the context of transgenic plants, will be required to elucidate the function of the proteins reported here in viral movement. However, our ability to demonstrate protein–protein interactions via two independent yeast or plant-based assays provides strong support for which host factors should be pursued in future studies.

Our model for transport of rhabdoviral nucleocapsids is distinctly different from that of Huang and associates (2005), who proposed that P3-mediated movement of RYSV required direct binding of P3 to nucleocapsids. However, these authors only investigated binding between P3 and N, whereas we have considered all pairwise interactions for SYN-1 proteins, except for L. Additionally, our model requires integration of data that demonstrates binding of sc4 to G (this study) and the report that sc4 co-purifies with membrane-associated nucleocapsids but not with purified nucleocapsids treated with organic solvents to remove membranes (Scholthof et al. 1994). It is possible that different rhabdoviruses, particularly those with dicot versus monocot hosts, could use different mechanisms for cell-to-cell transport. In this regard, this study strongly implicates cytoplasm-tethered transcription factors in the cell-to-cell movement of plant-adapted rhabdoviruses, a finding that heretofore has not been reported.

Transcription factors that are tethered in the cytoplasm are membrane associated (Chen et al. 2008; Kim et al. 2010) and are typically members in the basic leucine zipper and NAC (NAM/ATAF1-2/CUC2) transcription factor families, which include AtVOZ1, Ni67, and sc4i21. At present, known cyto-

plasm-tethered transcription factors utilize transmembrane anchors to mediate their retention in the ER and are released from these membranes upon proteolytic cleavage. Ni67 is ER associated but lacks a predicted transmembrane domain or signal peptide, whereas sc4i21 is microtubule associated. This suggests that there may be multiple mechanisms to retain transcription factors outside of the nucleus. This contention is supported by the reports from animal and plant systems that implicate microtubules in transcriptional activation by NF κ B and RNA processing (Hamada et al. 2009; Jackman et al. 2009). Consistent with the localization of Ni67, AtVOZ fused to tagRFP, accumulated in ER-associated complexes in *N. benthamiana* leaves (B.-E. Min and M. Goodin, unpublished data). It is curious that the related proteins Ni67 and sc4i21 have such different localization patterns, the reasons for which will be investigated in future studies.

Taken together, the reported two-hybrid screens provided high-confidence interactions that were all positive in independent binding assays with their corresponding bait proteins. More importantly, all the associated localization studies returned biologically relevant information that fit logically into a model for cell-to-cell movement of nucleorhabdoviruses. Thus, there should be high confidence and strong support for preparing comprehensive protein interaction and localization maps for genetically diverse viruses, in a manner similar to the generation of transcriptional profiles (Whitham et al. 2003; Senthil et al. 2005; Dardick 2007; Ascencio-Ibáñez et al. 2008). Collectively, these data will be critical for systems biology approaches to understanding plant–virus interactions (Mendez-Rios and Uetz 2010).

MATERIALS AND METHODS

Plant materials and plasmid.

Wild-type and transgenic *N. benthamiana* plants expressing fluorescent marker proteins targeted to the nucleus, ER, or actin filaments were maintained in the greenhouse under ambient conditions (Martin et al. 2009). SYN-1 was maintained in a similar manner in *N. benthamiana*. The marker construct for highlighting microtubules (GFP-MBD) was described by Marc and associates (1998).

Isolation of total RNA, reverse-transcriptase polymerase chain reaction.

Total RNA was extracted from plant tissues using the RNeasy plant minikit (Qiagen, Basel, Switzerland) according to the manufacturer's instructions. Except where noted, first-strand cDNA synthesis and polymerase chain reactions (PCR) were carried out using Superscript reverse transcriptase III (Invitrogen, Carlsbad, CA, U.S.A.) and Phusion high-fidelity DNA polymerase (Finnzymes, Espoo, Finland), respectively. To allow full-length host proteins to introduce into Gateway-compatible vectors, PCR reaction was conducted by gene-specific primers flanked by the *attB* sequence.

High-resolution yeast two-hybrid screens.

The coding sequences for the sc4, M, and N proteins of SYN-1 were PCR amplified and cloned into pB27 as a C-terminal fusion to LexA (N-LexA-bait-C) and into pB66 as a C-terminal fusion to the Gal4 DNA-binding domain (N-Gal4-bait-C). The constructs were checked by sequencing the entire insert and used as a bait to screen a random-primed *N. benthamiana* cDNA library constructed into pP6. pB27, pB66, and pP6 derive from the original pBTM116 (Vojtek and Hollenberg 1995), pAS2 $\Delta\Delta$ (Fromont-Racine et al. 1997), and pGADGH (Bartel et al. 1993) plasmids, respectively. To maximize proteome representation, the relative percentage contributions of mRNA for library con-

Fig. 9. Host factor and viral protein interaction map for *Sonchus yellow net virus*. Proteins forming heterologous interactions are connected by a straight line. Self-interactions are indicated by curved arrows. The superscript indicates steady-state localization: MT = microtubule, ER = endoplasmic reticulum, m = membranes, n = nuclear, and p = cell periphery.

struction from the following sources were fully expanded leaves (50%), roots of mature plants (10%), whole 2-week-old seedlings (10%), leaves treated with salicylic acid (10%), and leaves infected with SYNV (10%) or an RNA silencing mutant of *Tomato bushy stunt virus* (10%). The random-primed cDNA library contains an average insert size of 800 nucleotides and a complexity of 50 million independent fragments in *Escherichia coli* and 10 million independent fragments in yeast. To ensure exhaustive and reproducible Y2H results, the library was screened to saturation using an optimized cell-to-cell mating procedure using a Y187 (mata) and L40DGal4 (mata) yeast strains as previously described (Fromont-Racine et al. 1997) for the LexA constructs and Y187 (mata) and CG1945 (mata) yeast strains for the Gal4 constructs. On average, 108 million interactions per screen were tested, corresponding to approximately 10-fold coverage of the library.

For the N protein, 79.3 million and 63.6 millions interactions were tested in screens conducted with pB27 and pB66, respectively. For the M protein, 76.6 million and 365 million interactions were tested in screens conducted with pB27 and pB66, respectively. For the sc4 protein, 85.6 million and 82.8 million interactions were tested in screens conducted with pB27 and pB66, respectively. The prey fragments of the positive clones were amplified by PCR and sequenced at their 5' and 3' junctions. The resulting sequences were used to identify the corresponding interacting proteins in the GenBank database (National Center for Biotechnology Information [NCBI]) using a fully automated procedure. A confidence score (predicted biological score) was attributed to each interaction as previously described (Formstecher et al. 2005). Interactors for further study were selected based on confidence scores (A or B), our ability to recover full-length clones, and the fact that

Fig. 10. Model of the *Sonchus yellow net virus* (SYNV) cell-to-cell movement complex. Sc4i21 and sc4i17 are a microtubule-anchored transcription activator and motor-kinesin, respectively. Ni67 is an endoplasmic reticulum (ER)-associated transcription activator that may shuttle into the nucleus where it interacts with the SYNV-N protein and, presumably SYNV, nucleocapsids. Mi7 is also a transcription factor that interacts with the M protein, and which is also likely a nuclear shuttle protein. Current data suggest that Mi7 and Ni67 could function cooperatively to export nucleocapsids from the nucleus to establish a movement complex with sc4 and G or G* in the cytoplasm. Nucleocapsids that are not exported bud through the inner nuclear membrane to form mature virions that accumulate in the perinuclear space. Based on electron microscopy data, it is likely that most nucleocapsids undergo morphogenesis (bold arrow) given that the movement complex has not yet been observed or isolated.

the recombinant proteins could be expressed efficiently as fusions to autofluorescent proteins.

DNA sequence analysis.

Assembly of nucleotide sequences into full-length sequences encoding each interactor was performed using the DNASTAR v.7 software package. Homology searches by various BLAST tools were conducted on the NCBI server. ORF were identified using an ORF finder search tool (T. Tatusov and R. Tatusov, NCBI, Bethesda, MD, U.S.A.). The deduced amino acid sequences of proteins encoded by host genes were analyzed using a variety of algorithms provided by the ExPASy proteomics server (Gasteiger et al. 2003), including Compute PI/MW (Bjellqvist et al. 1993), PSORT for prediction of protein localization (Nakai and Kanehisa 1991), SignalP for prediction of signal peptide cleavage sites (Bendtsen et al. 2004), NetN-Glyc for prediction of N-glycosylation sites (Blom et al. 2004), and NetNES for prediction of nuclear export signals (la Cour et al. 2004).

Protein expression in plant cells.

Protein expression in plant cells for protein localization or bimolecular fluorescence complementation was conducted essentially as described by Bandyopadhyay and associates (2010). Briefly, sequence-validated full-length clones in vector pDONR221 (Invitrogen) and pDONRzeo (Invitrogen) of all relevant ORF were recombined into appropriate binary vectors for the expression of autofluorescent protein fusions in plant cells for localization and BiFC assays using a variety of pSITE or pSITEII vectors (Chakrabarty et al. 2007; Goodin et al. 2007; Martin et al. 2009). Vectors employed in this study were pSITE-2CA (GFP fusions) and pSITEII-6C1 (TagRFP fusions) for localization experiments and the pSITE-BiFC-nEYFP and pSITE-BiFC-cEYFP vectors for BiFC assays. BiFC assays were conducted as described for production of a protein interaction map for PYDV (Bandyopadhyay et al. 2010). Recombinant vectors were transformed into *Agrobacterium tumefaciens* LBA4404. Agroinfiltration for expression of protein fusions in plant cells was conducted essentially as described previously (Goodin et al. 2005). Each expression construct was examined in sections taken from a minimum of three leaves from each of three separate plants (nine leaves total). Several hundred cells were examined for each experiment and at least three high-resolution micrographs were acquired for each construct.

For expression of recombinant proteins containing an amino-terminal FLAG tag, we converted pSAT6-FLAG (gift from Tzvi Tzfira) to its pSITE equivalent as described by Martin and associates (2009). Cloning and expression from this vector, pSITE-FLAG, were as described above.

Laser scanning confocal microscopy.

All microscopy was performed with an Olympus FV1000 laser-scanning confocal microscope as described previously (Goodin et al. 2005). BiFC assays were conducted as described for production of a protein interaction map for PYDV, as described by Bandyopadhyay and associates (2010). Briefly, all proteins were tested as fusions to the amino (nec) and carboxy (cec) terminal portions of yellow fluorescent protein. GST or MBP served as nonbinding controls.

Drug treatments for depolymerization of tubulin and actin.

Stock solutions of latrunculin B (Lat B; Sigma, St. Louis) and oryzalin (Fluka) were made in concentrations of 10 and 20 mM in dimethyl sulfoxide, respectively. Then, 100 μ M oryzalin or 10 μ M LatB were co-infiltrated with tagRFP fusions to host proteins. Disruption of actin and tubulin by drugs was confirmed by confocal microscopy at 2 days postinfiltration.

Construction of pNIA

for nuclear import assays in yeast cells.

Recombinant pNIA (Bandyopadhyay et al. 2010) plasmids for expression of host proteins, Mi7, Sc4i17, Sc4i21, and Ni67, as well as genes for MBP and histone 2B, were transformed into *Saccharomyces cerevisiae* L40 (Zaltsman et al. 2007). The transformed yeast cells were grown for 4 days at 30°C on minimal media lacking tryptophan (Trp⁻). Yeast colonies were then restreaked onto minimal media lacking both tryptophan and histidine (His⁻) containing 50 mM 3-amino-1,2,4-triazole. Growth of yeast cultures on Trp⁻ or His⁻ media was indicative of a functional nuclear localization signal in proteins expressed from pNIA (Zaltsman et al. 2007).

Immunodetection of proteins.

Tissue to be prepared for immunodetection was sampled from *N. benthamiana* leaves with a number 8 cork borer (1-cm i.d.). Protein extracts were prepared in a 1.5-ml Eppendorf tube by grinding three leaf discs in 200 μ l of 2 $\times$ sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer (0.5 M Tris-HCl [pH 6.8], 10% SDS, 7.5% glycerol, 5% β -mercaptoethanol, and 0.05% Bromophenol Blue) and heated in a boiling water bath (100°C) for 5 min. Proteins were separated by discontinuous SDS-PAGE using 12% gels. Following electrophoresis, gels were developed using PageBlue dye to stain total proteins (Fermentas Life Sciences, Vilnius, Lithuania) or subjected to Western immunoblot analysis after transfer of the proteins to nitrocellulose membranes. Antibodies for SYN-V-G raised in mouse (Goldberg et al. 1991) or M2 anti-flag antibody (Sigma) were used for detection. Immunoblots were developed using a colorimetric assay employing the appropriate secondary antibody conjugated to alkaline phosphatase with nitro-blue tetrazolium chloride and 5-bromo-4-chloro-3'-indolylphosphate *p*-toluidine.

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