The Nucleolar Fibrillarin Protein is Required for Helper Virus-Independent Long-Distance Trafficking of a Subviral Satellite RNA in Plants

Chih-Hao Chang\textsuperscript{a,b}, Fu-Chen Hsu\textsuperscript{b}, Shu-Chuan Lee\textsuperscript{b}, Yih-Shan Lo\textsuperscript{b}, Jiun-Da Wang\textsuperscript{b}, Jane Shaw\textsuperscript{c}, Michael Taliansky\textsuperscript{c}, Ban-Yang Chang\textsuperscript{d}, Yau-Heiu Hsu\textsuperscript{e}, and Na-Sheng Lin\textsuperscript{a,b,1}

\textsuperscript{a}Institute of Plant Biology, National Taiwan University, Taipei, Taiwan 11106
\textsuperscript{b}Institute of Plant and Microbial Biology, Academia Sinica, Taipei, Taiwan 11529
\textsuperscript{c}The James Hutton Institute, Invergowrie, Dundee, United Kingdom DD2 5DA
\textsuperscript{d}Department of Biochemistry, National Chung Hsing University, Taichung, Taiwan 40227
\textsuperscript{e}Graduate Institute of Biotechnology, National Chung Hsing University, Taichung, Taiwan 40227
\textsuperscript{1}Corresponding author: nslin@sinica.edu.tw

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The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantcell.org) is: Na-Sheng Lin (nslin@sinica.edu.tw)

One sentence summary: Bamboo mosaic virus satellite RNA can move autonomously in a fibrillarin-dependent manner in the absence of its helper virus in \textit{Nicotiana benthamiana}. 

ABSTRACT

RNA trafficking plays pivotal roles in regulating plant development, gene silencing, and adaptation to environmental stress. Satellite RNAs (satRNAs), parasites of viruses, depend on their helper viruses (HVs) for replication, encapsidation, and efficient spread. However, it remains largely unknown how satRNAs interact with viruses and the cellular machinery to undergo trafficking. Here, we show that the P20 protein of Bamboo mosaic potexvirus satRNA (satBaMV) can functionally complement in trans the systemic trafficking of P20-defective satBaMV in infected Nicotiana benthamiana. The transgene-derived satBaMV, uncoupled from HV replication, was able to move autonomously across a graft union identified by RT-qPCR, northern blot and in situ RT-PCR analyses. Co-immunoprecipitation experiments revealed that the major nucleolar protein fibrillarin is co-precipitated in the P20 protein complex. Notably, silencing fibrillarin suppressed satBaMV-, but not HV-, phloem-based movement following grafting or co-inoculation with HV. Confocal microscopy revealed that the P20 protein co-localized with fibrillarin in the nucleoli and formed punctate structures associated with plasmodesmata. The mobile satBaMV RNA appears to exist as ribonucleoprotein (RNP) complex composed of P20 and fibrillarin, whereas BaMV movement proteins, capsid protein, and BaMV RNA are recruited with HV co-infection. Taken together, our findings provide insight into movement of satBaMV via the fibrillarin–satBaMV–P20 RNP complex in phloem-mediated systemic trafficking.

INTRODUCTION

RNA trafficking is essential for plant development, nutrient allocation, gene silencing, and stress responses (Lucas et al., 2001; Kehr and Buhtz, 2008; Turgeon and Wolf, 2009; Ursache et al., 2014). For efficient trafficking, plants have evolved complex networks of regulatory components that enable local and long-distance communication (Middleton et al., 2012). While systemic trafficking is enabled by phloem transport, local cell-to-cell communication relies on microchannels that traverse plant cell walls, known as plasmodesmata (PD) (Lucas et al., 2009; Kragler, 2013). Companion cell-sieve element PDs mediate the selective trafficking of RNAs through the phloem translocation stream (Aoki et al., 2005; Kehr and Buhtz, 2008; Turgeon and Wolf, 2009; Ursache et al., 2014). Recent findings on the movement of
small RNAs, including microRNAs and small interfering RNAs (siRNAs) (Dunoyer et al., 2010; Molnar et al., 2010), have greatly advanced our understanding of the intercellular signaling that coordinates gene expression during development.

RNAs can assemble with various proteins into ribonucleoprotein (RNP) complexes during cell-to-cell or long-distance trafficking through the phloem (Haywood et al., 2005; Bailey-Serres et al., 2009; Ham et al., 2009; Pallas and Gomez, 2013). Long-distance trafficking has been shown to be mediated by the binding of RNA to the RNA-interacting domains of phloem-mobile RNA-binding proteins (RBPs) (Ham et al., 2009; Pallas and Gomez, 2013). For example, phloem RBP50 binds to the polypyrimidine-tract binding motif of GA-INSENSITIVE PHLOEM RNA (Ham et al., 2009) for phloem-mediated trafficking. In addition, phloem RBPs can selectively bind to small RNAs, as well as mRNAs or viral RNAs, to mediate their trafficking (Aoki et al., 2005; Kehr and Buhtz, 2008; Ham et al., 2009; Hipper et al., 2013). Thus, phloem RBPs are translocated with plant RNAs and are likely to be important determinants of plant RNA vascular trafficking (Lucas et al., 2001; Aoki et al., 2005; Kehr and Buhtz, 2008; Ham et al., 2009; Turgeon and Wolf, 2009; Pallas and Gomez, 2013).

Viral spread from infected cells to neighboring cells requires that the PD size exclusion limits be increased through the action of viral movement protein (MP) (Gopinath and Kao, 2007; Canetta et al., 2008; Harries et al., 2009; Hipper et al., 2013). Changes in PD permeability are also thought to enable movement into the vascular system during systemic phloem-mediated trafficking. Thus, viruses must have evolved various strategies to interact with cellular factors to be loaded into and unloaded from the vascular system (Chen et al., 2000; Kim et al., 2007; Harries et al., 2009; Raffaele et al., 2009; Taliansky et al., 2010; Semashko et al., 2012; Hipper et al., 2013). Host factors such as myosin are required for MP targeting to and virus
movement through the PD (Amari et al., 2014; Harries et al., 2009); intercellular and long-distance trafficking of *Tobacco mosaic virus* (TMV) (Chen et al., 2000) and *Groundnut rosette virus* (GRV) (Kim et al., 2007; Taliansky et al., 2010; Semashko et al., 2012) were substantially delayed in plants silenced for *PECTIN METHYLESTERASE* and *fibrillarin*. However, remorin, a Solanaceae protein resident in membrane rafts and plasmodesmata, can interact physically with the MP from *Potato virus X* (PVX) and negatively regulates PVX intercellular and phloem-mediated trafficking (Raffaele et al., 2009). Some viral RNAs and viroids also contain important 3D RNA motifs required for intercellular movement (Takeda et al., 2011).

Satellite RNAs (satRNAs) are parasites of RNA viruses that are almost exclusively associated with plant viruses. These entities lack appreciable sequence similarity to the genomes of their helper viruses (HVs), but depend on HV-encoded proteins for replication and encapsidation (Hu et al., 2009). The mechanisms by which satRNAs undergo intracellular or intercellular trafficking and whether satRNA transport depends on HV replication remain unknown.

*Bamboo mosaic virus* (BaMV)-associated satRNA (satBaMV) has a single-stranded positive-sense RNA genome of ~835 nt (Lin and Hsu, 1994). SatBaMV encodes a 20 kDa nonstructural RNA-binding protein (P20) that is dispensable for replication (Lin et al., 1996) but is required for long-distance satBaMV transport in HV co-infected *Nicotiana benthamiana* (Palani et al., 2006; Vijayapalani et al., 2012). The P20 protein has several MP features, including RNA-binding activity (Tsai et al., 1999), strong self-interactions, and efficient cell-to-cell movement (Palani et al., 2006). P20 accumulates in the cytoplasm and nuclei of HV and satBaMV co-infected cells (Palani et al., 2009) and can be phosphorylated to negatively regulate the formation of
satBaMV RNP complexes (Vijayapalani et al., 2012). BaMV HV is a member of the Potexvirus genus that contains a single-stranded, positive-sense RNA genome with five open reading frames (ORFs). These ORFs encode a replicase, three MPs (TGBp1, TGBp2, TGBp3, encoded by a triple gene block), and a capsid protein (CP) (Lin et al., 1994), each required for cell-to-cell movement (Lin et al., 2004; Lin et al., 2006; Lan et al., 2010; Lee et al., 2011; Wu et al., 2011; Chou et al., 2013). However, the factors involved in long-distance trafficking of BaMV remain to be determined.

Here we provide the evidence that trafficking of P20-defective satBaMV can be restored in transgenic plants expressing P20 protein. Grafting experiments performed to uncouple the replication and trafficking events of satBaMV showed that satBaMV RNA alone can move systemically across a graft union into non-satBaMV-expressing tissue. Co-immunoprecipitation (co-IP) analysis revealed that a nucleolar protein, fibrillarin, was associated with the P20 protein complex. Thus it can be suggested that movement of satBaMV requires P20 interaction with fibrillarin to form mobile satBaMV RNP complexes. TGBp1, TGBp2, TGBp3, and CP were also present in the fibrillarin–satBaMV–P20 RNP complex after co-infection with satBaMV and HV, indicating that viral proteins also play important roles in satRNP complex trafficking.

RESULTS

The P20 Protein Plays a Crucial Role in SatBaMV Systemic Movement in N. benthamiana

Previously, we showed that the trafficking of P20-defective satBaMV exhibits low efficiency in N. benthamiana co-infected with BaMV HV (Lin et al., 1996; Palani et al., 2006; Vijayapalani et al., 2012). To provide further insights into the role of P20 in the systemic movement of satBaMV, we generated transgenic N. benthamiana
expressing the P20 protein under the phloem companion cell–specific SUC2 promoter (Fig. 1A). Immunoblot analysis confirmed that P20 was expressed in all 20 transgenic lines; we selected homozygous lines 1-29 and 3-1 for this study (Fig. 1A). No abnormal phenotypes were observed for SUC2 promoter-driven P20 transgenic N. benthamiana plants. To verify the localization of SUC2 driven P20, we created the construct SUC2pro:P20:eGFP for transient assays. Confocal microscopy clearly showed that P20 expressed from the SUC2pro:P20:eGFP construct was present in the phloem along the leaf vein of agro-infiltrated leaves whereas it localized to almost all cell types when it was expressed from 35Spro:P20:eGFP (Figure 1B).

For complementation assays, we first generated P20-defective satBaMV. The P20 gene of pCBSF4, the full-length cDNA clone of satBaMV (Lin et al., 2004), was replaced with GFP to generate a P20-defective satBaMV reporter plasmid (pCBSGFP); plants were mechanically inoculated with this plasmid and the BaMV infectious cDNA clone, pCB (Lin et al., 2004). BSGFP RNA accumulation was observed in the inoculated leaves of both wild type (WT) and P20-transgenic lines, but BSGFP RNA was not detected in the upper uninoculated leaves of WT plants (Fig. 1C, lane 4; Fig. 1C and Supplemental Figure 1A). Notably, a significant amount of BSGFP RNA (about 50% compared to WT BSF4 satBaMV in the WT inoculated leaves) was detected in the upper uninoculated leaves of two independent P20 transgenic lines at 20 days post-inoculation (DPI) from 4 independent experiments, each involving 4 plants (Fig. 1C, lane 8; Fig. 1D and Supplemental Figure 1A). To further confirm the accumulation of BSGFP RNA in BaMV co-infected WT or P20 transgenic line 1-29, we performed confocal microscopy to visualize the accumulation of BSGFP protein after co-infection of N. benthamiana with pCB. Long-distance trafficking of BSGFP RNA from inoculated leaves to the systemic leaves was rescued.
in the P20 transgenic line 1-29 (Supplemental Figure 1B-d), which suggests that P20
in trans significantly contributes to long-distance trafficking of BSGFP RNA.

**HV-Independent Long-Distance Trafficking of SatBaMV in N. benthamiana**

SatBaMV depends entirely on HV for replication, encapsidation, and efficient
spread in plants (Lin and Hsu, 1994). To determine whether satBaMV can move
systemically in the absence of HV, we generated five 35S promoter-driven satBaMV
transgenic N. benthamiana lines. The satBaMV RNA of the transgene was expressed
in all N. benthamiana tissues, including root, stem, leaves and flowers, in lines 2-6
and 9-2 (Fig. 2A). No abnormal phenotypes were observed in the 35S
promoter-driven satBaMV transgenic N. benthamiana plants.

To determine whether satBaMV RNA alone can move systemically, we grafted
satBaMV-transgenic N. benthamiana onto WT N. benthamiana and vice versa via
cleft grafting (Figs. 2B to 2D; Supplemental Figure 2). As shown in Figure 2B, the
leaves L7 and L8 were detached before grafting, whereas leaves L6, L9, and L10
were harvested at 12 days after grafting (DAG) and L11 at 15 DAG. SatBaMV was
detected in transgenic and WT leaves from chimeric grafts regardless of whether the
WT plants served as scions or stocks (Fig. 2B; Supplemental Figure 2A). When
satBaMV transgenic lines served as scions and WT as stocks, satBaMV RNA and P20
protein were detected in all assayed leaves from L6 to L11; however, satBaMV RNA
and P20 protein were detected only in L6 and L9 when satBaMV transgenic lines
served as stocks and WT as scions (Fig. 2B). Quantitative analyses of four
independent experiments revealed that satBaMV RNA was undetectable in L10 and
L11 by RNA gel blot (Supplemental Figure 2A). Moreover, quantitative RT-PCR
(RT-qPCR) detected that accumulation of satBaMV progressively decreased in stem,
petiole and leaf L9 while satBaMV trafficked from satBaMV transgenic stocks to WT
scion with levels about 82%, 41% and 14%, respectively, of those in L6 of the stocks (Supplemental Figure 3A). Our grafting experiments indeed showed that satBaMV RNA can move long distance alone across the graft union.

To investigate satBaMV long-distance trafficking in the presence of HV, L6 or L9 leaves near the graft union were co-agroinfiltrated with the HV infectious cDNA clone pKB (Liou et al., 2013) at 9 DAG. As expected, satBaMV RNA accumulated in the leaves of both scions and stocks of satBaMV transgenic and WT plants after grafting with HV infection at 12 DAG (Supplemental Figure 2C, lanes 5-8).

To further determine whether satBaMV can undergo trafficking without HV in response to source-to-sink strength, we detached mature and young leaves (L7-L11) and left only the newly emerged youngest leaf L12 after grafting onto the scions (Fig. 2C). RNA gel blot (Fig. 2C), RT-PCR (Supplemental Figure 2B) and RT-qPCR (Supplemental Figure 3B) analyses revealed no satBaMV RNA accumulation in L12 of WT grafted onto satBaMV transgenic lines, nor was there P20 protein accumulation based on immunoblot analysis (Fig. 2C). However, satBaMV RNA and P20 protein were detected in L6 of WT stocks supported by satBaMV transgenic scions from 4 independent experiments (Fig. 2C; Supplemental Fig. 2B; Supplemental Figure 3B).

Since satBaMV could not move to the L12 (Fig. 2C), we examined whether dark treatment could enhance satBaMV long-distance trafficking, because previous studies have indicated that dark treatment may alter the source–sink relationship and increase virus susceptibility (Lemonie et al., 2013; Helms and McIntyre, 1967). Scions were subjected to dark treatment for 3 days before the leaves and stems were harvested for analysis (i.e., dark treatment started at 12 DAG), and RNA or protein was then extracted from L6 to L14 and shoot apex (SA) for analysis by RT-PCR or
immunoblot, respectively; stem tissues were blotted onto nitrocellulose membrane by tissue blotting (Lin et al., 1990) at 15 DAG. RT-PCR or immunoblot analysis detected satBaMV or P20 protein accumulation in L12, L13, and SA after dark treatment but not in leaves without dark treatment (Fig. 2D, upper panel). Stem tissues were harvested between L6 and L12 at 15 DAG (Fig. 2D, lower panel). Tissue blotting further confirmed satBaMV RNA accumulation in WT scion stems under dark treatment, even in young stems close to L12. By contrast, only stem samples derived near L6 or L9 showed positive signals for satBaMV; satBaMV RNA failed to be detected in the upper stems near L10~L12 without dark treatment (Fig. 2D, lower panel; Supplemental Figure 3C). Therefore, dark treatment increased the long-distance trafficking of satBaMV to young leaves and stems, as well as to the SAs of WT plants (Fig. 2D; Supplemental Figure 3C).

To determine whether other RNA is also mobile, we grafted WT plants onto transgenic *N. tabacum* plants expressing the satRNA of *Cucumber mosaic virus* (satCMV), or *N. benthamiana* expressing BaMV CP or GFP (16c) (Brigneti et al., 1998) as controls. However, none of these transgene RNAs was detectable in WT scions at 15 DAG (Fig. 2E). These results indicate that satBaMV differs from satCMV in being dispensable for HV in systemic trafficking.

**In Situ Localization of SatBaMV**

To verify the phloem transportation and cell-to-cell movement of satBaMV, we used a modified method of in situ RT-PCR (Yoo et al., 2004). In situ RT-PCR allows the fluorescence associated with satBaMV to be visualized under confocal microscopy (Fig. 3A-L). We carried out the grafting experiment as for Figure 2B. Fresh sections were obtained from grafting stems, petioles and leaves of WT scions at 12 DAG. In situ RT-PCR revealed strong fluorescence in the stem (Fig. 3A-B),
petiole (Fig. 3C-D) and leaf (Fig. 3E-F) of satBaMV transgenic stock, including pith, phloem, cortex, epidermis, parenchyma, and mesophyll cells, with relatively lower fluorescence in xylem. The fluorescence associated with satBaMV was mainly detected in phloem and some in the cortex of the WT scion stems (Fig. 3G-H). In WT scion petioles, fluorescence was particularly located in internal phloem, external phloem and parenchyma cells (Fig. 3I-J). In addition, strong fluorescence was detected in phloem and parenchyma cells of the major veins of WT scion leaves, with little in the mesophyll and xylem (Fig. 3K-L), indicating that satBaMV was able to move cell-to-cell from phloem and parenchyma to mesophyll cells in WT scion leaves.

SIEVE ELEMENT OCCLUSION 1 (SEO1) mRNA was detected as an internal control for in situ RT-PCR and was found only in phloem (Fig. 3M-N), which is consistent with previous promoter assay findings (Ernst et al., 2012). Furthermore, we used another control, TOBACCO POLYPHENOL OXIDASE 1 (TobP1), established as a flower-specific gene (Goldman et al., 1998); TobP1 mRNA was not detected in N. benthamiana stem tissues (Fig. 3O-P). The absence of signal within stem and tissues in experiments with reverse transcriptase omitted from the reaction for satBaMV established the specificity of the protocol used in these studies (Fig. 3Q-R). Taken together, these studies confirm that satBaMV can move from cell to cell and long distance via phloem.

**Host Factors Immunoprecipitated with P20 Protein**

Since satBaMV is always associated with BaMV in the natural environment, we examined the host factors involved in movement of satBaMV in the presence of HV. Therefore, we used HV and satBaMV co-infected systemic leaves at 7 DPI to search for possible host factors involved in trafficking of satBaMV.
We performed co-IP experiments and analyzed the precipitated products by gel electrophoresis followed by liquid chromatography tandem mass spectrometry (LC-MS/MS). To avoid interference from Rubisco, the most abundant protein in leaves, we prepared Rubisco-depleted fractions and then incubated those with anti-P20 or pre-immune IgG, followed by Protein A Sepharose CL-4B. After washing, bound proteins were eluted and fractionated by gel electrophoresis. In mock-inoculated samples or in controls with pre-immune IgG, only small numbers of non-specific proteins were detected in silver-stained gels (Fig. 4B). By contrast, numerous proteins were resolved after anti-P20 IgG precipitation (Fig. 4B). LC-MS/MS analysis revealed several peptide sequences associated with anti-P20 co-immunoprecipitates after HV and satBaMV co-infection (Table 1). In three independent experiments, P20 was observed in band 4, which migrated slightly slower than the 17-kDa standard; this band was absent in co-IP complexes from total proteins of co-infected leaves treated with pre-immune IgG (Fig. 4B), which indicates the specificity of the anti-P20 IgG preparation in the co-IP experiments. As shown in Table 1, the most abundant viral CP was detected in band 3, whereas TGBp1 was in band 2. With the exception of Rubisco, which remained enriched in band 1, the most abundant host protein precipitating with anti-P20 IgG was a nucleolar protein, fibrillarin, which was detected in band 2 in three independent experiments (Table 1). Fibrillarin is known to interact with viral MPs and is important for long-distance trafficking of several RNA viruses (Kim et al., 2007; Taliantsky et al., 2010; Semashko et al., 2012; Zheng et al., 2015). The LC-MS/MS results were confirmed by immunoblot analysis, revealing the presence of P20 and fibrillarin in the P20 co-IP complex in the HV co-infected tissues (Figs. 4C and 4D).
As shown in Figure 2B-D, satBaMV systemic trafficking could be HV-independent; therefore, we also verified the satBaMV movement complex in the absence of HV. The WT scion *N. benthamiana*, grafted onto satBaMV transgenic line 2-6 (Fig. 4E), was harvested at 15 DAG and proteins were precipitated with anti-P20 IgG. In three independent experiments, P20 was detected in band 8 and fibrillarin was also detected in band 7 independent of HV by LC-MS/MS (Table 2). In summary, with or without HV, P20 and fibrillarin may form a complex *in vivo*.

**Fibrillarin Silencing Suppresses Long-Distance Trafficking of SatBaMV**

To examine whether fibrillarin has a role in satBaMV trafficking in the absence of HV, we used virus-induced gene silencing (VIGS) with a *Tobacco rattle virus* (TRV) vector (Ratcliff et al., 2001) to reduce *fibrillarin* expression in *N. benthamiana* plants. *PHYTOENE DESATURASE* (PDS), the silencing of which is reflected by photobleaching of leaves, was used as a control. Agroinfection with pTRV-NbFib carrying a fragment of the *fibrillarin* gene from *N. benthamiana* (Kim et al., 2007) resulted in 60% reduction in levels of both *fibrillarin* mRNA and protein in VIGS plants (Fig. 5A). RNA gel blot analysis revealed that the satBaMV RNA was no longer detectable in *fibrillarin*-silenced scion plants after grafting onto the satBaMV transgenic line at 9 DAG (Fig. 5A).

To further confirm that satBaMV systemic movement without HV depends on fibrillarin, we grafted the *N. benthamiana fibrillarin* knockdown line (Shaw et al., 2014) onto satBaMV transgenic plants. RNAi knockdown of *coilin*, encoding one of the main components of Cajal bodies (Ogg and Lamond, 2002), served as a control. SatBaMV did not move into *fibrillarin* RNAi scions, but substantial movement was evident in the *coilin* RNAi scions at 9 DAG (Fig. 5B); quantitative analysis revealed that satBaMV mRNA was greatly reduced in *fibrillarin* RNAi scions but not *coilin*.
RNAi scions (Fig. 5C). Thus, this result supports the crucial role of fibrillarin in satBaMV long-distance transport without HV.

To examine the effect of fibrillarin on satBaMV co-infection with HV, we agroinfiltrated *N. benthamiana* plants with pKB (Liou et al., 2013) and pKF4 (Liou et al., 2013), carrying the infectious cDNA clones of BaMV and satBaMV, respectively. Although mRNA expression of coilin and fibrillarin was greatly suppressed in infiltrated and upper uninfiltrated leaves of RNAi *N. benthamiana* plants (Fig. 5B and data not shown), the inoculated leaves of WT, coilin, or fibrillarin RNAi plants contained similar levels of BaMV or satBaMV RNA accumulation after infiltration with BaMV or co-infiltration with HV+satBaMV at 5 DPI (Fig. 5D). Notably, satBaMV RNA was greatly decreased in the upper leaves of fibrillarin RNAi plants (Fig. 5E, lanes 21-24 vs 5-8) but not coilin RNAi plants (Fig. 5E, lanes 13-16 vs 5-8; Supplemental Figure 4B) co-agroinfiltrated with pKB and pKF4. Quantitative analyses of three independent experiments revealed that satBaMV accumulation in the upper, non-infiltrated leaf L4 of fibrillarin RNAi plants was about 20% of that of the WT or coilin RNAi plants but undetectable in L5 of fibrillarin RNAi plants (Supplemental Figure 4B). SatBaMV accumulation in L4 or L5 of WT and coilin RNAi plants did not differ (Supplemental Figure 4B). However, BaMV accumulation was not greatly affected in the upper non-infiltrated leaves (L4-L7) among the WT, fibrillarin RNAi, or coilin RNAi plants after BaMV or satBaMV co-infection at 15 DPI (Fig. 5E; Supplemental Figure 4A). Therefore, fibrillarin silencing impaired only the long-distance trafficking of satBaMV in HV and satBaMV co-infected plants.

**Fibrillarin and P20 Form Complexes with SatBaMV RNA**

The previous results suggest that fibrillarin may be important for long-distance trafficking of satBaMV during BaMV co-infection. Therefore, we performed co-IP
assays to determine the nature of the putative protein–protein interactions. Total proteins were extracted from the leaves of *N. benthamiana* plants infected with HV or co-infected with satBaMV, and co-IP assays were then performed with anti-P20 or anti-fibrillarin IgG. Input proteins and co-IP fractions were confirmed by immunoblot analysis (Fig. 6).

With anti-P20 IgG, protein samples extracted from leaves co-infected with HV and satBaMV revealed signals for P20, fibrillarin, TGBp1, TGBp2, and CP (Fig. 6A, lane 6). These proteins were not detected in samples from plants infected with HV alone or in healthy control leaves (Fig. 6A, lanes 2 and 4). Likewise, co-IP assay with anti-fibrillarin IgG revealed equivalent signals for P20, fibrillarin, TGBp1, TGBp2, and CP in HV and satBaMV co-infected leaves (Fig. 6B, lane 6), indicating that fibrillarin interacted directly or indirectly with P20, TGBp1, TGBp2, and CP. To determine whether fibrillarin interacts with P20 directly, we generated fusions of activation domain (AD) or binding domain (BD) with fibrillarin or P20 for yeast two-hybrid assays. We observed strong self-interactions for both P20 and fibrillarin and also a direct interaction between fibrillarin and P20, regardless of which protein was fused to AD (Supplemental Figure 6). This interaction is consistent with the results obtained by co-IP assay with anti-P20 or anti-fibrillarin IgG.

Like potexviruses, all three TGBps and CP are required for BaMV cell-to-cell and systemic movement (Lin et al., 2004; Lin et al., 2006; Lan et al., 2010; Lee et al., 2011; Wu et al., 2011; Chou et al., 2013). To examine the interaction of TGBp3 with P20 and/or fibrillarin, we inoculated *N. benthamiana* leaves with a 35S promoter-driven HV derivative (pCB-P3HA) carrying a TGBp3::HA fusion (Fig. 6C, Chou et al., 2013) with or without the satBaMV pCBSF4 plasmid (Lin et al., 2004). Co-IP assays with an HA antibody precipitated TGBp3HA, TGBp1, TGBp2, and CP...
but not P20 or fibrillarin in fractions, from pCB-P3HA-infected plants (Fig. 6C, lane 4). The HA antibody additionally precipitated fibrillarin and P20 from protein extracts of plants co-inoculated with pCB–P3HA and pCBSF4 (Fig. 6C, lane 5) but not from uninoculated WT, pCB-inoculated, or pCB and pCBSF4 co-inoculated plants. However, co-IP assays with anti-P20 IgG (Fig. 6D) or anti-fibrillarin IgG (Fig. 6E) revealed positive signals for TGBp3HA in HV and satBaMV co-infected leaves (Figs. 6D and Fig. 6E, lane 6). Hence, consistent with observations in other potexviruses (Park et al., 2013), BaMV TGBps and CP may interact with each other, and P20 may form protein complexes with fibrillarin, BaMV TGB proteins and CP in plant tissues co-infected with HV and satBaMV.

To further confirm that the P20–fibrillarin protein complexes are RNP complexes, we extracted RNA from total sap and co-IP fractions after incubation with anti-P20 or anti-fibrillarin IgG. In plants co-infected with HV and satBaMV, both HV and satBaMV RNAs were present in the total sap and co-IP fractions, as shown by using anti-P20 (Fig. 6F, lanes 5-6 and 11-12) or anti-fibrillarin IgG (Fig. 6G, lanes 5-6 and 11-12). In the negative control (immunoprecipitation of total sap of HV-infected plants by using anti-P20 IgG), HV RNA was detected in only total sap from HV-infected plants but not in co-IP fractions with anti-P20 or anti-fibrillarin IgG (Figs. 6F and 6G, lanes 3-4). These results support the conclusion that P20 can form RNP complexes with fibrillarin and also complexes with HV proteins as well as HV and satBaMV RNAs in satBaMV co-infected plants.

However, WT scions grafted onto the satBaMV transgenic stock, 2-6 line, without HV, showed satBaMV RNA in co-IP fractions with anti-P20 or anti-fibrillarin IgG for the formation of a satBaMV-P20-fibrillarin RNP complex (Fig. 6H).
The Fibrillarin–SatBaMV–P20 RNP Complex is Absent from the Fibrillarin RNAi Transgenic Line

To verify whether the composition of the satBaMV–P20 RNP complex with or without HV was changed by reduced fibrillarin level, the fibrillarin RNAi plants were grafted onto satBaMV transgenic lines 2-6 (Supplemental Figure 5A). Fibrillarin RNAi scion tissues were harvested and proteins were precipitated with anti-P20 IgG. In three independent experiments, P20 was detected in band 7; other detected proteins were chloroplast, ribosomal and helicase proteins (Supplemental Table 1). Total proteins were extracted from the leaves of fibrillarin RNAi plants coinfected with HV and satBaMV, and co-IP assays were performed with anti-P20 IgG at 15 DPI (Supplemental Figure 5B). The most abundant P20 was detected in band 9, whereas viral CP and TGBp1 co-migrated into band 8 (Supplemental Table 2), and ATP synthase, pyrophosphorylase 2, glycine decarboxylase P-protein 2, and ribosomal and helicase proteins were detected in three independent experiments. These data show that the composition of the satBaMV–P20 RNP complex with and without HV was distinct in plants with reduced fibrillarin levels. Furthermore, no fibrillarin was detected in the satBaMV–P20 RNP complex in fibrillarin RNAi transgenic line (Supplemental Table 1 and 2), suggesting that the residual fibrillarin produced in the RNAi line is completely or nearly completely used for its primary function in ribosome biogenesis.

P20 Co-localizes with Fibrillarin and Forms Punctate Structures at the Cell Periphery

To reveal the subcellular localization of P20, we constructed an Agrobacterium-compatible plasmid, pBin-P20-eGFP, for transient expression of GFP-tagged P20. P20-eGFP was located in the nucleus and also at the cell periphery,
where it formed punctate structures (Fig. 7A-a), as described previously (Palani et al., 2006; 2009). At higher magnification, P20-eGFP could be seen to form one or two foci in the nucleus (Fig. 7B-a to c). To determine whether P20-eGFP co-localized with fibrillarin (FIB2), we agro-infiltrated leaves with pBin-mCherry-NbFIB2 alone or with pBin-P20-eGFP. Transiently expressed mCherry-NbFIB2 was mainly localized in the nucleolus (Fig. 7B-d to f). When P20-eGFP was co-expressed with mCherry-NbFIB2, these two proteins exhibited perfect co-localization in the nucleolus (Fig. 7B-g to i), indicating that P20 can be imported into the nucleus and become enriched in the nucleolus.

P20 protein was previously found to move cell-to-cell autonomously (Palani et al., 2006); accordingly, we examined whether the P20 peripheral punctae were associated with the PD. We used both DsRed-tagged TMV MP (Fig. 7C-a to f) and aniline blue staining (Fig. 7C-g to l) as PD markers. Regardless of which PD marker was used, most, if not all, of the P20-eGFP co-localized with labeled PD.

To further determine whether the P20-eGFP localized in the PD channel, we used leaves expressing P20-eGFP and TMVMP-DsRed plasmolyzed with 1 M NaCl. Nearly half of the leaf cells were plasmolyzed after NaCl treatment. A large amount of P20-eGFP localized along the shrunken cell membrane. However, substantial P20-eGFP still remained in the cell wall and co-localized with the PD marker TMVMP-DsRed after plasmolysis (Fig. 7D). Taken together, these findings suggest that P20 can localize to the nucleolus with fibrillarin and form punctate structures at the cell periphery with integration into the PD channel.

**DISCUSSION**

Although satRNAs are subviral agents that require HVs for efficient replication and long-distance trafficking in co-infected plants, our data clearly show that transgenic
satBaMV alone can move systemically across a graft union in *N. benthamiana*. We also demonstrated that expression of P20 in phloem (Fig. 1B) can complement long-distance trafficking of P20-defective satBaMV in *P20*-transgenic *N. benthamiana* (Figs. 1C and 1D; Supplemental Figure 1). In addition, autonomous satBaMV trafficking into WT scions is fibrillarin-dependent (Fig. 5). In the absence of HV, satBaMV RNA appears to exist as an RNP complex composed of P20 and fibrillarin (Fig. 4E and Table 2), whereas in the presence of HV, viral MPs and CP are also recruited into the mobile RNP complex for efficient trafficking (Fig. 6). Therefore, satBaMV trafficking appears not to require prior replication of satBaMV with HV or encapsidation by HV CP (Lin and Hsu, 1994), and the mobility of satBaMV can be independent of replication. This observation implies that satBaMV may have an advantage for survival in nature because its capacity for autonomous trafficking to the distal leaves may enhance its chances to encounter HV for further amplification and spread once satBaMV and HV initially infect different cells. Fibrillarin is required for only satBaMV, but not HV long-distance trafficking, which suggests that HV and satRNA may have evolved distinct methods for trafficking. A previous study also found a differential requirement for the host factor heat shock protein 90 in the replication of HV and satBaMV (Huang et al., 2012).

Viroids do not encode any translatable products and are not encapsidated. Viroid RNA alone can replicate and traffic efficiently without the need for an HV in host plants (Flores et al., 2009). However, differentiating between replication and movement of viroids through their interactions with cellular factors is problematic. Although replication-independent long-distance trafficking of *Brome mosaic virus* RNA3 can be recapitulated by agroinfection of individual cDNA components into different expression sites in *N. benthamiana*, the detection of the movement signal of
RNA3 requires the subsequent replication of RNA1 and RNA2 (Gopinath and Kao, 2007). Thus, uncoupling of satBaMV replication from trafficking will provide an opportunity to gain greater molecular insights into satRNA systemic movement in planta.

Unlike most satRNA-encoded proteins required for satRNA replication (Hu et al., 2009), the satBaMV-encoded P20 non-structural protein assists satBaMV long-distance trafficking in plants (Lin et al., 1996; Palani et al., 2006; Vijayapalani et al., 2012) (Fig. 1). Previously, we showed that P20 can preferentially bind to the 5’- and 3’- UTRs of satBaMV to form satBaMV–P20 RNP complexes (Tsai et al., 1999; Vijayapalani et al., 2012), and that formation of the complexes is negatively regulated by P20 phosphorylation (Vijayapalani et al., 2012). Our current results show that P20 can form punctate structures localized at PD (Fig. 7) and the satBaMV–P20 RNP complexes can traffic autonomously through the phloem in satBaMV-transgenic stocks or scions (Figs. 2B-D). In situ RT-PCR experiments also provided strong support that satBaMV moves within the functional phloem system. On grafting, the satBaMV is present in phloem of the stem, petiole and major vein of leaves in WT scions (Fig. 3). The accumulation pattern of visualized satBaMV within vascular tissues in N. benthamiana phloem resembles that of SEO1 in N. tabacum (Ernst et al., 2012). Thus, evidence in support of the satBaMV phloem trafficking was provided by the combination of grafting, RNA gel blot analysis, in situ RT-PCR and tissue blotting techniques. With these experimental approaches, we can conclude that the satBaMV is translocated through satBaMV transgenic stock into the WT scion via the phloem. This finding agrees with other recent studies of cellular or viral RNAs indicating that there are systemic recombination signals, that small RNAs alone can undergo systemic transport across a graft union (Turgeon and Wolf, 2009; Dunoyer et
al., 2010), and that grafting is able to determine the specificity and efficiency of RNA trafficking (Kehr and Buhtz, 2008). We found that GFP RNA, BaMV CP RNA, and CMV satRNA in transgenic lines were all restricted to stocks (Fig. 2E). Therefore, satBaMV may contain long-distance trafficking determinants that involve specific RNA sequences or structural elements, and the P20 protein may interact with host factors.

Using a combination of co-IP and LC-MS/MS along with VIGS assays or the fibrillarin RNAi transgenic line, we further determined that the HV-independent or -dependent systemic movement of the satBaMV RNP complexes depends on fibrillarin (Fig. 5; Supplemental Figure 5; Supplemental Tables 1-2). Fibrillarin is required for ribosomal RNA processing (Barneche et al., 2000; Saez-Vasquez et al., 2004) and is a nucleolar-localized RBP required for systemic infection of GRV (Kim et al., 2007; Canetta et al., 2008), Potato leafroll virus (Haupt et al., 2005), and Rice stripe tenuivirus (Zheng et al., 2015). Nucleolar co-localization of fibrillarin and P20 protein (Fig. 7), along with detection of fibrillarin, P20 protein, and satBaMV RNA in co-IP complexes (Fig. 4 and 6) but not in the fibrillarin RNAi transgenic line (Supplemental Figure 5; Supplemental Table 1-2), is consistent with the evidence for interaction of fibrillarin with satBaMV–P20 RNP complexes (Figure 4E and Table 2). Experiments with transgenic RNAi lines reinforced our findings that fibrillarin is crucial for systemic movement of satBaMV but not BaMV trafficking (Fig. 5E). Hence, fibrillarin is the identified host factor that is differentially required for satRNA and HV long-distance trafficking. Our results also suggest that satBaMV and BaMV may move independently by interacting with distinct cellular factors. Increasing evidence suggests that interactions between viral MPs and nuclear-localized proteins may represent an essential step for virus systemic trafficking (Solovyev and Savenkov, 2004).
2014), but determining where and how such interactions occur requires additional experimentation. Mobile satBaMV RNA complexes containing P20, fibrillarin, satBaMV RNA, TGBp1-3, and CP are also present with HV co-infection (Fig. 6). In this way, BaMV TGBps may participate in PD gating (Howard et al., 2004) or by interaction with fibrillarin–satBaMV–P20 RNP complex to help with satBaMV movement. Taken together, our findings for the fibrillarin–satBaMV–P20 RNP complex suggest that the nuclear/nucleolar-localized fibrillarin activity may involve cytosolic interactions that require a high degree of coordination of the P20 protein of satBaMV during phloem-mediated trafficking.

Phloem-mobile RNP complexes can move in the translocation stream from source to sink tissues (Ursache et al., 2014). This RNP complex transport pathway can be regulated when plants respond to environmental cues or pathogen attack (Pallas and Gomez, 2013; Ursache et al., 2014). Dark treatment may alter the source–sink relationship and increase virus susceptibility (Lemonie et al., 2013; Helms and McIntyre, 1967). Our results demonstrate that dark treatment facilitates long-distance trafficking of fibrillarin-based satBaMV RNP complexes, presumably via a change in photoassimilate allocation. Several studies have also indicated that fibrillarin can exit the nucleoli and move to other cell compartments during exposure to biotic or abiotic stresses, such as actinomycin D (Chen and Jiang, 2004), mercury treatment (Chen et al., 2002), or GRV infection (Kim et al., 2007). Hence, fibrillarin may function in defense responses under stress conditions. However, whether P20 co-moves with fibrillarin or whether the nucleolar activity of P20 results in the relocalization of fibrillarin to the cytoplasm during satBaMV transport through the phloem remains to be determined.
In addition to identifying fibrillarin, we identified other host proteins, such as histone H3, which is crucial for trafficking of a Geminivirus DNA through the nuclear pore complex and PD (Zhou et al., 2011), in our co-IP experiments (Table 1). These proteins may also interact with P20, directly or indirectly, to form fibrillarin-based satBaMV RNP complexes. Whether the phloem-mobile satBaMV RNP complexes contain any phloem proteins or RNA-specific chaperones that could modify the satBaMV RNA structure, thereby facilitating systemic spread, requires further investigation.

METHODS

Construction of Plasmids

The gene encoding the satBaMV P20 protein was placed under the control of the SUC2 promoter in companion cells (Haywood et al., 2005) by inserting the SUC2 promoter at the HindIII/XbaI sites of p1390 (Haywood et al., 2005) to generate p1390-SUC2pro. The P20 gene was then inserted at the XbaI/BamHI sites of p1390-SUC2pro vector to generate SUC2pro:P20 for transformation. To generate SUC2pro:P20-eGFP for transient expression P20, the P20-eGFP DNA fragment was amplified from pCass-P20-EGFP (Palani et al., 2006) with the primers P20-eGFP-XbaI-F and P20-eGFP-BamHI-R (Supplemental Table 3), then cloned in the p1390-SUC2:P20 plasmid at the XbaI/BamHI sites.

To express P20-eGFP in N. benthamiana, we constructed pBIN-P20-eGFP. The P20-eGFP DNA fragment was amplified from pCass-P20-EGFP (Palani et al., 2006) using primers Tf-XmaI-F and Tf-XmaI-R (Supplemental Table 3), and then cloned into the pBIN61 plasmid at the XmaI site to generate pBIN-P20-eGFP. For transient expression of mCherry-NbFIB2 in N. benthamiana, we constructed
pBIN-mCherry-NbFIB2. The mCherry DNA fragment was first amplified from pBA-mCh-p1 (Chou et al., 2013) using primers mCherry-SmaI-F and mCherry-KpnI-R (Supplemental Table 3), and then cloned into the pCass plasmid (Ding et al., 1995) at the SmaI and KpnI sites to generate pCass-mCherry. Then, the FIB2 coding region was amplified from *N. benthamiana* cDNA using primers NbFIB2-KpnI-F and NbFIB2-EcoRI-R (Supplemental Table 3). The amplified FIB2 fragment was then cloned into pCass-mCherry to generate pCass-mCherry-NbFIB2. Finally, pBIN-mCherry-NbFIB2 was generated by amplifying the mCherry-NbFIB2 fragment from pCass-mCherry-NbFIB2 using primers Tf-Xmal-F and Tf-Xmal-R (Supplemental Table 3), and then cloning it into the pBIN61 vector at the Xmal site.

To construct the pBIN-TMVMP-DsRed plasmid required as a PD marker, we first amplified the DsRed DNA fragment from pdNR (Addgene) using primers DsRed KpnI-F and DsRed EcoRI-R (Supplemental Table 3); the fragment was then cloned into the pCass plasmid at the KpnI and EcoRI sites to generate pCass-DsRed. The DNA of TMVMP was amplified from pTMV-ΔCP using primers TMV-MP-StuI-F and TMV-MP-KpnI-F (Supplemental Table 3), and then cloned into the pCass-DsRed plasmid at the StuI and KpnI sites to generate pCass-TMVMP-DsRed. Finally, the DNA of TMVMP-DsRed was amplified from pCass-TMVMP-DsRed using primers Tf-Xmal-F and Tf-Xmal-R, and cloned into the pBIN61 plasmid at the Xmal site to generate pBIN-TMVMP-DsRed.

**Generation of Transgenic Plants**

The procedures for transformation and regeneration of transgenic *SUC2*<sub>pro</sub>.*P20* *N. benthamiana* plants were previously described (Lin et al., 2013). The transgenic lines were selected based on their resistance to 50 mg/L hygromycin on MS agar medium (Sigma-Aldrich Co. LLC). Seedlings exhibiting resistance to hygromycin were
examined for P20 gene expression by RT-PCR using primers P20-EcoRI-F and P20-EcoRI-R (Supplemental Table 3). In total, we obtained 20 independent homozygous lines after segregation analysis. The homozygous lines 1-29 and 3-1 were used in this study. Transgenic *N. benthamiana* lines expressing satBaMV driven by the 35S promoter were a gift from Dr. Yau-Heiu Hsu (National Chung Hsing University, Taichung, Taiwan). Five homozygous lines were obtained and lines 2-6 and 9-2 were used for grafting experiments. The transgenic *N. tabacum* lines expressing satCMV driven by the 35S promoter was a gift from Dr. Yau-Heiu Hsu (National Chung Hsing University, Taichung, Taiwan).

*Fibrillarin* RNAi transgenic *N. benthamiana* plants were generated by transformation with *A. tumefaciens* LBA4404 carrying the plasmid pFGC5941.Fibrillarin3’, as described for *coilin* RNAi plants (Shaw et al., 2014). The plasmid consisted of a 321-bp fragment (region 501-821) of the *N. benthamiana* fibrillarin gene (AM269909) cloned in opposite orientations flanking the *CHALCONE SYNTHASE* intron. Three independent *N. benthamiana* fibrillarin RNAi lines were selected and were found to elicit an approximately 60% reduction in fibrillarin expression. None of the lines exhibited obvious phenotype alterations, and Line #1 was used in this study.

*Coilin* RNAi transgenic *N. benthamiana* plants were previously characterized (Shaw et al., 2014). Transformation and regeneration of fibrillarin and coilin RNAi transgenic plants were as previously described (Taliansky et al., 2004). To avoid the possibility of “off-target” silencing, we confirmed that the fibrillarin and coilin fragments used in the RNAi constructs did not contain any 21-nt stretches showing similarity to other genes by using the siRNA scan website (http://bioinfo2.noble.org/RNAiScan.htm).
Protein Analysis

Leaves of SUC2pro:P20 transgenic plants, or those of BaMV- and satBaMV-infected plants, were ground in liquid nitrogen and resuspended in extraction buffer (50 mM Tris-HCl, pH 8, 10% glycerol, 1 mM EDTA, 100 mM NaCl, 1 mM PMSF). For co-IP, 20-fold diluted protein samples were used for immunoblot analysis with anti-P20, anti-TGBp1, anti-TGBp2, anti-HA (Chou et al., 2013), rabbit anti-actin (1/5000 dilution, provided by Y.-Y. Hsu) or anti-CP antibodies (Lin et al., 1992; Chou et al., 2013) together with anti-fibrillarin IgG, H-140 (sc-25397) (Santa Cruz Biotech), followed by staining with goat anti-rabbit IgG HRP secondary antibody (Abcam).

Plant Growth, Inoculation, and Grafting

All WT and transgenic N. benthamiana or N. tabacum plants were grown at 28°C in a walk-in plant growth chamber under a 16 h light/8 h dark cycle with a white light (Philips TLD 36W/840 ns) intensity of 185~222 μmol m⁻² s⁻¹ at the leaf surface. For each set of experiments, we used 4-week-old plants for inoculation. The methods for inoculation by Agrobacterium expressing BaMV or satBaMV were described previously (Liou et al., 2013).

WT or grafted N. benthamiana plants were agroinfected with Agrobacterium expressing full-length infectious cDNA clones of BaMV, pKB (Liou et al., 2013) or satBaMV, pKF4 (Liou et al., 2013) in pKYLX7 (Schardl et al., 1987) binary vector. For complementation assays, WT and SUC2pro:P20 transgenic plants were inoculated with 0.5 μg pCB alone, or co-inoculated with 0.5 μg pCBSF4 or pCBSGFP (Lin et al., 2004; Vijaypalani et al., 2012).

Grafting was performed as described (Turnbull et al., 2002) except that approximately 40-day-old N. benthamiana or N. tabacum plants were used for cleft
grafting. Each grafting experiment was repeated at 4 times, each including 4 plants.

**Agrobacterium Culture, Infiltration, and Virus-Induced Gene Silencing (VIGS)**

Plasmids (pKB or pKF4) for protein transient expression were transformed into *Agrobacterium* C58C1 by electroporation. *Agrobacterium* cultures were grown as described (Liou et al., 2013) and diluted to an optical density of 0.4-1.0 at 600 nm for infiltration into leaves of *N. benthamiana* plants. For co-expression of two plasmids, two solutions of *Agrobacterium*, each harboring a specific plasmid, were mixed in a 1:1 ratio prior to agro-infiltration. *Agrobacterium* was infiltrated into the intercellular space of *N. benthamiana* leaves.

For VIGS assays, 4-week-old plants of *N. benthamiana* were infiltrated with mixture of *A. tumefaciens* LBA4404 harboring either the TRV1 or TRV2 RNA2 vector (p0704) containing the *fibrillarin* fragment (Kim et al., 2007). In parallel, silencing of the *PHYTOENE DESATURASE* (PDS) leading to a photobleached phenotype was used as a marker for monitoring the effectiveness of VIGS. At 7 days after agroinfiltration, we harvested systemically infected leaves for RNA accumulation assays.

**RNA Analysis**

Total RNA was extracted from *N. benthamiana* or *N. tabacum* tissues using Tripure in accordance with the manufacturer's instructions (Roche). RNA gel blot analysis was carried out as described previously (Lin et al., 1996). BaMV and satBaMV accumulation was analyzed using $^{32}$P-labeled RNA probes specific for the BaMV 3’ end generated from *Hind*III-linearized pBaHB (Lin et al., 1993) and specific for full-length satBaMV generated from *Eco*RI-linearized pBSHE (Lin et al., 2013), respectively. The satCMV probe was transcribed from *Hind*III-linearized pGEM4 (provided by Dr. Yau-Heiu Hsu) using SP6 RNA polymerase. The GFP probe was
transcribed from EcoRI-linearized pGEM-T Easy GFP using SP6 RNA polymerase (New England Biolabs) (Vijayapalani et al., 2012). Accumulation of fibrillarin, coilin and actin mRNA was conducted as described by Kim et al. (2007). Four independent replicates were performed for each experiment.

For RT-qPCR, 2 μg total RNA extracted from plants was reverse-transcribed into poly d(T) cDNA by using SuperScript III reverse transcriptase (Invitrogen) in triplicates with the GeneAmp 9700 sequence detection Real-Time PCR system (Life Technologies) and SYBR Green I core reagent (Life Technologies). Normalization of satBaMV accumulation was to the 18S gene. Primers for satBaMV (satBaMV<sup>rL</sup>-F and satBaMV<sup>rL</sup>-R) and 18S (18S-F and 18S-R) are in Supplemental Table 3.

To analyze BaMV or satBaMV RNA in the co-IP fractions, we extracted total RNA from the anti-P20 or anti-fibrillarin co-IP fractions followed by RT-PCR. Primer set BaMV-F and BaMV-R (Supplemental Table 3) was used to amplify a 729-nt BaMV cDNA 3’ fragment (produced after 25 cycles of PCR). Similarly, primers satBaMV-F and satBaMV-R (Supplemental Table 3) were used to amplify full-length satBaMV cDNA.

**Tissue Blotting**

Sections were cut from fresh stem tissues by hand with a new razor blade. Tissue blots were made by pressing the newly cut surface onto a membrane; a <sup>32</sup>P-labeled RNA probe specific for full-length satBaMV was generated from EcoRI-linearized pBSHE (Lin et al., 2013), and then used to detect the distribution of satBaMV in the tissues (Lin et al., 1990).

**In Situ RT-PCR**

The localization of satBaMV was determined by using established protocols for in situ RT-PCR (Lee et al., 2004). A reverse-transcriptase cocktail (containing
SuperScript III reverse-transcriptase components, and satBaMV-R primer [Supplemental Table 3] at 75 μM) was prepared immediately before use. Fresh 200-μm thick sections were obtained using a D.S.K. Microslicer DTK-1000. Sections were placed onto a glass slide, covered by a 25 μL aliquot of the above cocktail and sealed with ampicover discs and clips. The reverse-transcription step was performed at 50°C for 60 min. For the PCR step, the satBaMV-F primer of 75 μM (Supplemental Table 3) and ChromaTide Alexa Fluor 488-5-dUTP (20 μM; Molecular Probes, Eugene, OR) were added; dTTP was reduced to 10 μM. The amplification protocol consisted of 10 cycles; 30 s at 94°C, 30 s at 55°C and 60 s at 72°C. A MJ research PTC-200 Peltier Thermal cycler was used for these experiments.

After this reaction series, sections were incubated (1 min) in absolute ethanol, followed by rinsing in 1 mM EDTA, and then overnight washing (16 h), at 22°C, in this EDTA solution.

**Co-Immunoprecipitation (co-IP) and LC-MS/MS**

Plant total proteins were extracted using Tris-HCl buffer (Dharmsiri et al., 2005) from systemically infected leaves of *N. benthamiana* agro-infiltrated with pKB at 7 DPI or together with pKF4. Plant Rubisco was removed using Seppro® RuBisCO Spin Columns (Catalog Number SEP070, Sigma-Aldrich Co. LLC) in accordance with the manufacturer’s instructions.

*In vivo* co-IP experiments with anti-P20 (Palani et al., 2009) and anti-fibrillarin IgG, H-140 (sc-25397) (Santa Cruz Biotech) were performed as described (Dharmsiri et al., 2005). Briefly, plant extracts containing 1 mg protein were incubated with anti-P20 IgG (1:150 v/v) for 1 h at 4°C on a rotary shaker. Then, 20 μL Protein A agarose beads (GE Healthcare Life Science, Sweden) were added, and the mixture was incubated for 3 h at 4°C. After washing with Tris-HCl buffer (Dharmsiri et al.,
2005), the immunoprecipitate resuspended in 2X sample buffer (4% SDS, 20% Glycerol, 0.12 M Tris pH 6.8, and 10% β-mercaptoethanol) and separated on a NuPAGE Novex 4-12% Bis-Tris protein gel (Invitrogen Life Science Technologies).

Gels were silver-stained, and protein bands were then excised and digested with trypsin for analysis by LC-MS/MS (Lo et al., 2011). LC-MS/MS fragmented ions were used with Mascot (http://www.matrixscience.com/search_form_select.html) to search against the most recent Arabidopsis, BaMV, and satBaMV databases in the National Center for Biotechnology Information (NCBI).

**Yeast Two-Hybrid Assay**

Yeast two-hybrid assays were performed as recommended by the manufacturers of the GAL4 Two-Hybrid Phagemid Vector kits (Agilent Technologies, Inc. 2011). The full-length coding sequence of fibrillarin was generated using NbFIB2-EcoRI-F and NbFIB2-EcoRI-R, and P20 was generated using P20-EcoRI-F and P20-EcoRI-R (Supplemental Table 3). Fibrillarin or P20 was cloned into downstream of the GAL4 activation domain [AD] at EcoRI site or downstream of the GAL4 DNA binding domain [BD] at the EcoRI site. The rich medium yeast extract, peptone, dextrose (YPD) is most commonly used for growing yeast under nonselective conditions. To test interactions between fibrillarin and P20, we coexpressed the constructs in YRG-2 yeast cells, and selected by incubation in leucine-tryptophan-histidine medium at 28°C for 2-3 days until colonies appeared. Each experiment was repeated three times.

**Confocal microscopy**

To visualize satBaMV accumulation from *in situ* RT-PCR (Fig. 3), fresh tissues were examined under a Zeiss LSM880 laser scanning microscope with a 40x/1.2 W Korr UV-VIS-IR objective lens. Images were captured by using the ZEN software with Ex/Em: 488 nm/505-550 nm.
To visualize PD in leaf epidermal cells, we infiltrated aniline blue fluorochrome (Biosupplies) (0.1 mg/ml in water) into agro-infected leaves of *N. benthamiana*, and then immediately examined the leaves using a Zeiss LSM510 laser scanning microscope with a 40x/1.2 W Korr UV-VIS-IR objective lens (Fig. 7). Images were captured using the LSM510 software with filters for aniline blue fluorochrome (Ex/Em: 405 nm/480-510 nm), GFP ((Ex/Em: 488 nm/505-575 nm), and mCherry/DsRed ((Ex/Em: 543 nm/560–615 nm). All images were processed and cropped using Zeiss LSM Image Browser and Photoshop CS5 (Adobe).

The agro-infiltrated leaves expressing P20-eGFP and TMVMP-DsRed at 2 days after inoculation were used for plasmolysis studies (Fig. 7D). The leaf discs were treated with 1 M NaCl for 20 min before observation. Samples were scanned under a Zeiss LSM880 laser scanning microscope with a C-Apochromat 40x/1.2 W Korr FCS M27 objective lens. Images were captured by using ZEN2 software with the filters of GFP (EX/Em: 488 / 500-550 nm) and DsRed (EX/Em: 561 / 570-619 nm).

**Accession Numbers**

Sequence data from this article can be found in the GenBank/EMBL databases under accession numbers shown in Tables 1 and 2, and Supplemental Tables 1 and 2.

**Supplemental Data**

**Supplemental Figure 1.** Trans-Complementation of the Systemic Movement of P20-defective Satellite RNA of Bamboo Mosaic Virus (satBaMV) in P20 transgenic *N. benthamiana*.

**Supplemental Figure 2.** Systemic movement of SatBaMV with and without HV.

**Supplemental Figure 3.** RT-qPCR analysis of satBaMV mRNA accumulation in *N.
Supplemental Figure 4. BaMV and SatBaMV accumulation in *coilin* or *fibrillarin* RNAi transgenic lines and fibrillarin accumulation in *fibrillarin* VIGS plants.

Supplemental Figure 5. Identification of P20-interacting protein complex from grafting *N. benthamiana* *fibrillarin* RNAi leaves by co-immunoprecipitation (co-IP).

Supplemental Figure 6. Yeast two-hybrid analysis of interactions between fibrillarin and P20.

Supplemental Table 1. Proteins identified by LC-MS/MS after the immunoprecipitation of P20 IgG from *fibrillarin* RNAi scions grafted onto satBaMV transgenic stock.

Supplemental Table 2. Proteins identified by LC-MS/MS after the immunoprecipitation of P20 IgG from BaMV and satBaMV co-infected *fibrillarin* RNAi plants.

Supplemental Table 3. List of primer sequences used in this study.

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AUTHOR CONTRIBUTIONS

C.-H.C., N.-S.L., and Y.-H.H. designed the project; C.-H.C. and Y.-S.L. conducted most of the experiments; S.-C.L. and J.-D.W. obtained confocal microscope images; J.S., M.T., B.-Y.C., and Y.-H.H designed the viral constructs and characterized the transgenic lines; C.-H.C., F.-C.H, Y.-H.H, M.T., and N.-S.L. analyzed data; and C.-H.C., F.-C.H., M.T., and N.-S.L. wrote the paper.
FIGURE LEGENDS

Figure 1. Trans-Complementation of the Systemic Movement of P20-Defective Satellite RNA of Bamboo Mosaic Virus (satBaMV BSGFP) in P20 Transgenic N. benthamiana.

(A) Physical map of a SUC2 promoter-driven P20 expression plasmid and immunoblot analysis of P20 accumulation in leaves of SUC2pro-P20 transgenic N. benthamiana (lines 1-29 and 3-1). WT: wild-type plant. rP20: recombinant P20 protein purified from E. coli. CBB: Coomassie Brilliant Blue staining.

(B) P20-eGFP localization under SUC2 promoter- and 35S promoter-driven expression in WT N. benthamiana leaves at 3 days post-inoculation (DPI). Arrowheads represent the leaf midrib.

(C) Schematic maps of satBaMV infectious clones and RNA accumulation of WT satBaMV (BSF4) and P20-defective satBaMV (BSGFP) in WT and P20 transgenic N. benthamiana (line 1-29). Leaves of WT and P20-transgenic N. benthamiana were co-inoculated with pCB (Lin et al., 2004) and pCBSF4 (Lin et al., 2004) or pCBSGFP. Inoculated leaves (IL) were harvested at 10 DPI and uninoculated upper leaves (UL) at 20 DPI for RNA gel blot analysis of BaMV and satBaMV RNA accumulation. BaMV and satBaMV accumulation was detected by using 32P-labeled RNA probes specific for the BaMV 3’ end and satBaMV 3’-UTR, respectively.

(D) Quantitative analysis of satBaMV accumulation in WT and P20-transgenic line 1-29 from four independent biological samples, each involving four plants. Values are normalized against BSF4 satBaMV in inoculated leaves of WT plants. Data are mean±SD from four experiments and were analyzed by Student t test. Different letters indicate significant difference (p < 0.05).
Figure 2. HV-Independent Systemic Movement of SatBaMV.

(A) Physical map of pKF4 for generating satBaMV transgenic plants and satBaMV RNA accumulation in transgenic *N. benthamiana* lines 2-6 and 9-2 by RNA gel blot. r: root; s: stem; L1: 1st leaf; L2: 2nd leaf; L3: 3rd leaf; f: flower. rRNA of EtBr served as loading control. Four independent biological samples, each involving four plants, generated similar results.

(B, C) Illustrations of grafting experiments with 40-day-old WT and satBaMV-transgenic *N. benthamiana* (sat). SatBaMV RNA and P20 protein accumulation were examined by RNA gel blot and immunoblot analysis, respectively. rRNA and Coomassie blue staining were used for loading controls. Four independent biological samples, each involving four plants, generated similar results. (B) Leaf 7 (L7) and leaf 8 (L8) were detached before grafting. L6, L9, and L10 near the graft union were harvested at 12 days after grafting (DAG) and L11 at 15 DAG. (C) Leaves L7 to L11 were detached immediately after grafting; L6 and L12 were harvested at 15 DAG.

(D) RT-PCR and tissue blot detection of satBaMV RNA in grafting scions after dark treatment. The dark treatment of scions started at 12 DAG for 3 days. RNA and protein extracted from L6, L12-L14, and shoot apex (SA) were sampled at 15 DAG and examined by RT-PCR and immunoblot analysis, respectively. Plants without dark treatment (w/o dark treatment) were used as controls. rRNA and Coomassie blue staining were used for loading controls. The tissue blots from left to right were prepared from grafting stem tissues between L6 to L12, followed by hybridization with satBaMV-specific probe. Four independent biological samples, each involving four plants, generated similar results.

(E) Detection of transgene RNA in transgenic stocks and WT scions after grafting.
WT plants were grafted onto transgenic *N. benthamiana* expressing GFP (GFP) or BaMV capsid protein (CP) or transgenic *N. tabacum* expressing *Cucumber mosaic virus* satRNA (satCMV). RT-PCR analysis of mRNA level at 15 DAG. Four independent biological samples, each involving four plants, generated similar results.

**Figure 3. In situ RT-PCR Detection of SatBaMV.**

The grafting experiment was illustrated as in Fig. 2B. Stems and petioles between L6 and L9 were harvested at 12 DAG for *in situ* RT-PCR detection.

(A-F) Presence of satBaMV RNA in the stem (A, B), petiole (C, D) and leaf midrib (E, F) of satBaMV transgenic *N. benthamiana* stock.

(G-L) Presence of satBaMV RNA in the stem (G, H), petiole (I, J) and leaf midrib (K, L) of WT scion.

(M, N) Detection of *SIEVE ELEMENT OCCLUSION 1 (SEO1)* mRNA in the WT scion stem. *SEO1* mRNA was restricted to the sieve element (Ernst et al., 2012). Arrows in (M) indicate sieve element.

(O, P) Detection of *TOBACCO POLYPHENOL OXIDASE 1* mRNA in the WT scion stem, which was exclusively present in flower organs (Goldman et al., 1998), as a negative control.

(Q, R) Detection of satBaMV RNA without reverse transcriptase in the reaction in the WT scion stem as control for non-specific amplification during RT-PCR.

The results of *in situ* RT-PCR were observed by confocal laser scanning microscopy. Green signal represents incorporation of Alexa Fluor 488-labeled nucleotides during specific amplification of target genes indicated beside images and the respective merged image, with bright-field image in the right panel. Four independent biological samples, each involving four plants, of *in situ* RT-PCR detection generated similar
results. Scale bars: 100 μm. Pi, pith; Xy, xylem; Co, cortex; Ep, epidermis; Ph, phloem; IP, internal phloem; EP, external phloem; Pa, parenchyma; Me, mesophyll.

**Figure 4.** Identification of P20-interacting Protein Complex from Grafting *N. benthamiana* Leaves with HV-dependent or -independent SatBaMV Infection by Co-immunoprecipitation (co-IP).

(A) Coomassie blue staining of total protein extracted from leaves of healthy or BaMV (pKB) + satBaMV (pKF4) (Liou et al., 2013) co-infected *N. benthamiana* at 7 DPI.

(B) Co-IP protein complexes by pre-immune IgG (PIS) or anti-P20 IgG (P20) were separated by SDS-PAGE. Protein bands were visualized by silver staining; frames indicate protein bands excised for LC-MS/MS protein identification. The gel is representative of 3 independent experiments.

(C-D) Detection of P20 and fibrillarin in co-IP complex from anti-P20 IgG or PIS antibody. Input (-) and complex were separated by SDS-PAGE followed by immunoblot analyses with anti-P20 (C) or anti-FIB IgG (D).

(E) HV-independent grafting experiments are illustrated in the left as in Fig. 2B. Total protein was extracted from WT scion leaves after grafting at 15 DAG. Co-IP and protein analysis were performed as in (B).

**Figure 5.** Fibrillarin Silencing Suppresses SatBaMV Trafficking.

(A) Accumulation of satBaMV RNA and *fibrillarin* mRNA and protein in *N. benthamiana* leaves from satBaMV transgenic stock and grafted WT and TRV-induced *fibrillarin* silenced (Fib-s) scions. Plants were first infiltrated with *A. tumefaciens* strain LBA4404 carrying binary vectors expressing pTRV1 and pTRV2-fibrillarin (Fib-s); 7 days later, a fib-s scion was grafted onto satBaMV transgenic line 2-6 (sat). At 9 DAG, stock and scion leaves were harvested for RNA
gel blot and immunoblot analyses. CBB: Coomassie Brilliant Blue staining. Four independent biological samples, each involving four plants, generated similar results. *Actin* and CBB were used as loading controls.

(B) Accumulation of satBaMV RNA, *coilin* and *fibrillarin* mRNA and fibrillarin protein in *N. benthamiana* leaves from satBaMV transgenic stock and grafted WT, *fibrillarin* and *coilin* RNAi (Shaw et al., 2014) transgenic scions. RNA and protein analysis were performed at 9 DAG as described in (A). Four independent biological samples, each involving four plants generated similar results.

(C) Statistical analysis of satBaMV accumulation in grafted *coilin* or *fibrillarin* RNAi transgenic scions. Values are normalized against the WT sample. Data are mean ± SD from four independent biological samples, each involving four plants and were analyzed by Student t test. The asterisk represents significant difference between WT and *fibrillarin* RNAi lines (* = P < 0.001, ns = not significant).

(D, E) Accumulation of BaMV and satBaMV in WT, *coilin*, and *fibrillarin* RNAi transgenic plants. RNA gel blot analyses of BaMV and satBaMV RNAs in WT, *coilin* and *fibrillarin* RNAi transgenic plants agroinfected with pKB (B) or pKB + pKF4 (B+S) in inoculated leaves (IL) harvested at 5 DPI (D) and uninoculated upper leaves (UL) at 15 DPI (E). Four independent biological samples, each involving four plants generated similar results. “−”: uninoculated (healthy) plant control. rRNA: loading control.

**Figure 6.** Mobile SatBaMV–P20 Complexes Contain Fibrillarin (FIB), TGBp1, TGBp2, TGBp3, CP, BaMV, and SatBaMV RNAs in *N. benthamiana* Agroinfected with pKB and pKF4.

(A, B) WT *N. benthamiana* plants were agroinfected with pKB (B) or pKB + pKF4 (B+S). Healthy (H) leaves were used as a control. Total proteins were extracted from
uninoculated upper leaves of healthy plants or agroinfected plants at 15 DPI, and co-IP was performed with anti-P20 (A) or anti-fibrillarin (FIB) (B) IgG followed by protein A agarose immunoprecipitation. Input (−) and eluted (+) proteins were separated by SDS-PAGE followed by immunoblot analyses with anti-P20, anti-FIB, anti-TGBp1, anti-TGBp2 or anti-CP IgG. Lane 6 was loaded in 20-fold dilution. CBB indicates the input protein before co-IP. Four independent biological samples generated similar results.

(C) Genomic map of BaMV infectious clone pCB-P3HA (Chou et al., 2013). *N. benthamiana* plants were inoculated with WT pCB (B), pCB + pCBSF4 (B+S) or pCB-P3HA (B-P3HA), or pCB-P3HA + pCBSF4 (B-P3HA+S). Co-IP and immunoblot analyses were as described in (A, B), except that the HA antibody was used for immunoprecipitation. Input proteins before co-IP were detected by immunoblot against actin and CBB staining. Four independent biological samples generated similar results.

(D, E) Immunoblot analysis of TGBp3HA in co-IP complex. Co-IP with anti-P20 (D) or anti-FIB (E) IgG followed by protein A agarose. Input (−) and eluted (+) proteins were separated by SDS-PAGE followed by immunoblot analyses with anti-HA antibody. Lane 6 was loaded in 20-fold dilution. Four independent biological samples were examined.

(F, G) RT-PCR detection of BaMV (left panels) and satBaMV RNA (right panels) in co-IP fractions. RNA was extracted from anti-P20 (F) or anti-FIB (G) IgG co-IP fractions from healthy (H) or agroinfected with pKB (B) or pKB + pKF4 (B+S) *N. benthamiana* leaves. RT-PCR was used to detect the presence of BaMV and satBaMV RNAs. Amplified products were separated with agarose gel and the expected sizes of the BaMV (0.8 kb) and satBaMV (0.8 kb) fragments were shown. Four independent
biological samples generated similar results.

(H) RT-PCR detection of satBaMV RNA in co-IP fractions from non-grafted or grafted plants. The grafting experiment was illustrated in Fig. 2B. Total protein from non-grafted WT (lane 1) or grafted L6 (lane 2) and L9 (lane 3) leaves was extracted and used for co-IP at 15 DAG. RNA was extracted and detected by RT-PCR from anti-P20 or anti-FIB IgG co-IP fractions. Four independent biological samples were examined.

Figure 7. Subcellular Localization of satBaMV-Encoded Protein P20 and Fibrillarin in *N. benthamiana* Epidermal Cells. Proteins were fused to eGFP or DsRed/mCherry and expressed in *N. benthamiana* leaves by agro-infiltration. Two days after agro-infiltration, leaf sections were examined under a confocal laser scanning microscope.

(A) P20-eGFP localizes to the nucleus and cell periphery as punctate structures (a). The cell periphery is shown through the merging of the fluorescent signal with the bright field (b). (B) Localization of P20-eGFP and mCherry-NbFIB2 in nucleus and nucleolus. Epidermal cells expressing P20-eGFP (a-c) or mCherry-NbFIB2 (d-f), or co-expressing both proteins (g-i) were examined. The nuclear area is indicated by the dashed circle.

(C) Localization of P20-eGFP peripheral punctate adjacent to plasmodesmata. P20-eGFP co-expressed with TMVMP-DsRed (a-f) or callose deposition (as stained with aniline blue) (g-l). The images in the dashed square areas of (a-c) and (g-i) are shown magnified in (d-f) and (j-l), respectively.

(D) Localization of P20-eGFP after plasmolysis. The leaf tissues were plasmolyzed with 1 M NaCl before confocal microscopy. Images show localization of P20-eGFP (a) and TMVMP-DsRed (b) and merged images (c and d) in the plasmolyzed cell.
plasmolyzed region is labeled (Plasmolysis). Green, red and blue represent eGFP, DsRed/mCherry and aniline blue signals, respectively. Arrows and arrowheads indicate nucleus and nucleolus, respectively. Open triangles indicate the plasmodesmata. Scale bars: 40 μm in (A), 5 μm in (B) and (D), 10 μm in (C, a-c and g-i) and 2 μm in (C, d-f and j-l). Individual and merged images were edited using Photoshop CS5.
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Table 1. Proteins identified by LC-MS/MS after immunoprecipitation of P20 IgG from BaMV and satBaMV co-infected *N. benthamiana*.

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*: BaMV- or satBaMV-encoded proteins

Score is parameter characterizing identification reliability of a certain protein. In general, at score value > 23, identification is considered as reliable (p<0.05).
Table 2. Proteins identified by 1D LC-MS/MS after immunoprecipitation of P20 IgG from wild-type scion grafted onto satBaMV transgenic stock.

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*: satBaMV-encoded proteins

Score is parameter characterizing identification reliability of a certain protein. In general, at score value > 23, identification is considered as reliable (p<0.05).
Figure 1. Trans-Complementation of the Systemic Movement of P20-Defective Satellite RNA of *Bamboo Mosaic Virus* (satBaMV BSGFP) in P20 Transgenic *N. benthamiana*.

(A) Physical map of a SUC2 promoter-driven P20 expression plasmid and immunoblot analysis of P20 accumulation in leaves of *SUC2* pro::P20 transgenic *N. benthamiana* (lines 1-29 and 3-1). WT: wild-type plant. rP20: recombinant P20 protein purified from *E. coli*. CBB: Coomassie Brilliant Blue staining.

(B) P20-eGFP localization under SUC2 promoter- and 35S promoter-driven expression in WT *N. benthamiana* leaves at 3 days post-inoculation (DPI). Arrowheads represent the leaf midrib.

(C) Schematic maps of satBaMV infectious clones and RNA accumulation of WT satBaMV (BSF4) and P20-defective satBaMV (BSGFP) in WT and P20 transgenic *N. benthamiana* (line 1-29). Leaves of WT and P20-transgenic *N. benthamiana* were co-inoculated with pCB (Lin et al., 2004) and pCBSF4 (Lin et al., 2004) or pCBSGFP. Inoculated leaves (IL) were harvested at 10 DPI and uninoculated upper leaves (UL) at 20 DPI for RNA gel blot analysis of BaMV and satBaMV RNA accumulation. BaMV and satBaMV accumulation was detected by using 32P-labeled RNA probes specific for the BaMV 3’ end and satBaMV 3’-UTR, respectively.

(D) Quantitative analysis of satBaMV accumulation in WT and P20-transgenic line 1-29 from four independent biological samples, each involving four plants. Values are normalized against BSF4 satBaMV in inoculated leaves of WT plants. Data are mean±SD from four experiments and were analyzed by Student t test. Different letters indicate significant difference (p < 0.05).
Figure 2. HV-Independent Systemic Movement of SatBaMV.

(A) Physical map of pKF4 for generating satBaMV transgenic plants and satBaMV RNA accumulation in transgenic *N. benthamiana* lines 2-6 and 9-2 by RNA gel blot. r: root; s: stem; L1: 1st leaf; L2: 2nd leaf; L3: 3rd leaf; f: flower. rRNA of EtBr served as loading control. Four independent biological samples, each involving four plants, generated similar results.

(B, C) Illustrations of grafting experiments with 40-day-old WT and satBaMV-transgenic *N. benthamiana* (sat). SatBaMV RNA and P20 protein accumulation were examined by RNA gel blot and immunoblot analysis, respectively. rRNA and Coomassie blue staining were used for loading controls. Four independent biological samples, each involving four plants, generated similar results. (B) Leaf 7 (L7) and leaf 8 (L8) were detached before grafting. L6, L9, and L10 near the graft union were harvested at 12 days after grafting (DAG) and L11 at 15 DAG. (C) Leaves L7 to L11 were detached immediately after grafting; L6 and L12 were harvested at 15 DAG.

(D) RT-PCR and tissue blot detection of satBaMV RNA in grafting scions after dark treatment. The dark treatment of scions started at 12 DAG for 3 days. RNA and protein extracted from L6, L12-L14, and shoot apex (SA) were sampled at 15 DAG and examined by RT-PCR and immunoblot analysis, respectively. Plants without dark treatment (w/o dark treatment) were used as controls. rRNA and Coomassie blue staining were used for loading controls. The tissue blots from left to right were prepared from grafting stem tissues between L6 to L12, followed by hybridization with satBaMV-specific probe. Four independent biological samples, each involving four plants, generated similar results.

(E) Detection of transgene RNA in transgenic stocks and WT scions after grafting. WT plants were grafted onto transgenic *N. benthamiana* expressing GFP (GFP) or BaMV capsid protein (CP) or transgenic *N. tabacum* expressing *Cucumber mosaic virus* satRNA (satCMV). RT-PCR analysis of mRNA level at 15 DAG. Four independent biological samples, each involving four plants, generated similar results.
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**(M, N)** Detection of *SIEVE ELEMENT OCCLUSION 1 (SEO1)* mRNA in the WT scion stem. *SEO1* mRNA was restricted to the sieve element (Ernst et al., 2012). Arrows in (M) indicate sieve element.

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(B) Co-IP protein complexes by pre-immune IgG (PIS) or anti-P20 IgG (P20) were separated by SDS-PAGE. Protein bands were visualized by silver staining; frames indicate protein bands excised for LC-MS/MS protein identification. The gel is representative of 3 independent experiments.

(C-D) Detection of P20 and fibrillarin in co-IP complex from anti-P20 IgG or PIS antibody. Input (-) and complex were separated by SDS-PAGE followed by immunoblot analyses with anti-P20 (C) or anti-FIB IgG (D).

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Figure 5. *Fibrillarin* Silencing Suppresses SatBaMV Trafficking.

(A) Accumulation of satBaMV RNA and fibrillarin mRNA and protein in *N. benthamiana* leaves from satBaMV transgenic stock and grafted WT and TRV-induced fibrillarin silenced (Fib-s) scions. Plants were first infiltrated with *A. tumefaciens* strain LBA4404 carrying binary vectors expressing pTRV1 and pTRV2-fibrillarin (Fib-s); 7 days later, a fib-s scion was grafted onto satBaMV transgenic line 2-6 (sat). At 9 DAG, stock and scion leaves were harvested for RNA gel blot and immunoblot analyses. CBB: Coomassie Brilliant Blue staining. Four independent biological samples, each involving four plants generated similar results. Actin and CBB were used as loading controls.

(B) Accumulation of satBaMV RNA, coilin and fibrillarin mRNA and fibrillarin protein in *N. benthamiana* leaves from satBaMV transgenic stock and grafted WT, fibrillarin and coilin RNAi (Shaw et al., 2014) transgenic scions. RNA and protein analysis were performed at 9 DAG as described in (A). Four independent biological samples, each involving four plants generated similar results.

(C) Statistical analysis of satBaMV accumulation in grafted coilin or fibrillarin RNAi transgenic scions. Values are normalized against the WT sample. Data are mean ± SD from four independent biological samples, each involving four plants and were analyzed by Student t test. The asterisk represents significant difference between WT and fibrillarin RNAi lines (* = P < 0.001, ns = not significant).

(D, E) Accumulation of BaMV and satBaMV in WT, coilin, and fibrillarin RNAi transgenic plants. RNA gel blot analyses of BaMV and satBaMV RNAs in WT, coilin and fibrillarin RNAi transgenic plants agroinfected with pKB (B) or pKB + pKF4 (B+S) in inoculated leaves (IL) harvested at 5 DPI (D) and uninoculated upper leaves (UL) at 15 DPI (E). Four independent biological samples, each involving four plants, generated similar results. “−”: uninoculated (healthy) plant control. rRNA: loading control.
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(A, B) WT *N. benthamiana* plants were agroinfected with pKB (B) or pKB + pKF4 (B+S). Healthy (H) leaves were used as a control. Total proteins were extracted from uninoculated upper leaves of healthy plants or agroinfected plants at 15 DPI, and co-IP was performed with anti-P20 or anti-fibrillarin (FIB) IgG followed by protein A agarose immunoprecipitation. Input (−) and eluted (+) proteins were separated by SDS-PAGE followed by immunoblot analyses with anti-P20, anti-FIB, anti-TGBp1, anti-TGBp2 or anti-CP IgG. Lane 6 was loaded in 20-fold dilution. CBB indicates the input protein before co-IP. Four independent biological samples generated similar results.

(C) Genomic map of BaMV infectious clone pCB-P3HA (Chou et al., 2013). *N. benthamiana* plants were inoculated with WT pCB (B), pCB + pCBSF4 (B+S) or pCB-P3HA (B-P3HA), or pCB-P3HA + pCBSF4 (B-P3HA+S). Co-IP and immunoblot analyses were as described in (A, B), except that the HA antibody was used for immunoprecipitation. Input proteins before co-IP were detected by immunoblot against actin and CBB staining. Four independent biological samples generated similar results.

(D, E) Immunoblot analysis of TGBp3HA in co-IP complex. Co-IP with anti-P20 (D) or anti-FIB (E) IgG followed by protein A agarose. Input (−) and eluted (+) proteins were separated by SDS-PAGE followed by immunoblot analyses with anti-HA antibody. Lane 6 was loaded in 20-fold dilution. Four independent biological samples were examined.

(F, G) RT-PCR detection of BaMV (left panels) and satBaMV RNA (right panels) in co-IP fractions. RNA was extracted from anti-P20 (F) or anti-FIB (G) IgG co-IP fractions from healthy (H) or agroinfected with pKB (B) or pKB + pKF4 (B+S) *N. benthamiana* leaves. RT-PCR was used to detect the presence of BaMV and satBaMV RNAs. Amplified products were separated with agarose gel and the expected sizes of the BaMV (0.8 kb) and satBaMV (0.8 kb) fragments were shown. Four independent biological samples generated similar results.

(H) RT-PCR detection of satBaMV RNA in co-IP fractions from non-grafted or grafted plants. The grafting experiment was illustrated in Fig. 2B. Total protein from non-grafted WT (lane 1) or grafted L6 (lane 2) and L9 (lane 3) leaves was extracted and used for co-IP at 15 DAG. RNA was extracted and detected by RT-PCR from anti-P20 or anti-FIB IgG co-IP fractions. Four independent biological samples were examined.
Figure 7. Subcellular Localization of satBaMV-Encoded Protein P20 and Fibrillarin in N. benthamiana Epidermal Cells. Proteins were fused to eGFP or DsRed/mCherry and expressed in N. benthamiana leaves by agro-infiltration. Two days after agro-infiltration, leaf sections were examined under a confocal laser scanning microscope.

(A) P20-eGFP localizes to the nucleus and cell periphery as punctate structures (a). The cell periphery is shown through the merging of the fluorescent signal with the bright field (b).

(B) Localization of P20-eGFP and mCherry-NbFIB2 in nucleus and nucleolus. Epidermal cells expressing P20-eGFP (a-c) or mCherry-NbFIB2 (d-f), or co-expressing both proteins (g-i) were examined. The nuclear area is indicated by the dashed circle.

(C) Localization of P20-eGFP peripheral punctate adjacent to plasmodesmata. P20-eGFP co-expressed with TMVMP-DsRed (a-f) or callose deposition (as stained with aniline blue) (g-l). The images in the dashed square areas of (a-c) and (g-i) are shown magnified in (d-f) and (j-l), respectively.

(D) Localization of P20-eGFP after plasmolysis. The leaf tissues were plasmolyzed with 1 M NaCl before confocal microscopy. Images show localization of P20-eGFP (a) and TMVMP-DsRed (b) and merged images (c and d) in the plasmolyzed cell. The plasmolyzed region is labeled (Plasmolysis). Green, red and blue represent eGFP, DsRed/mCherry and aniline blue signals, respectively. Arrows and arrowheads indicate nucleus and nucleolus, respectively. Open triangles indicate the plasmodesmata. Scale bars: 40 μm in (A), 5 μm in (B) and (D), 10 μm in (C, a-c and g-i) and 2 μm in (C, d-f and j-l). Individual and merged images were edited using Photoshop CS5.


The Nucleolar Fibrillarin Protein is Required for Helper Virus-Independent Long-Distance Trafficking of a Subviral Satellite RNA in Plants
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