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16. Dl^{EC}-containing medium [Sang's M3; penicillin and streptomycin (50 U/ml)] of 16-hour cultures of 0.7 mM $CuSO_4$ induced Dl-S2 cells were concentrated five times with a Centricon C-30 (Amicon) (5× Dl^{EC}). N-S2 and nontransfected S2 cells were induced with 0.7 mM $CuSO_4$ for 16 hours in medium with 5% fetal calf serum. The cells were collected by centrifugation, washed once in serum-free medium with 1% bovine serum albumin (BSA), and resuspended at 2×10^6 cells per milliliter in M3 (1% BSA). Two hundred fifty microliters of cells were added to 100 μl of Dl^{EC} concentrate, raised to 500 μl with M3 (1% BSA), and incubated for 1 hour at room temperature on a rocking table at five oscillations per minute. The mixture was layered over a cushion of 20% sucrose, 20 mM tris-HCl, 150 mM NaCl, 2 mM $CaCl_2$, and 1% BSA (pH 7.4) in microfuge tubes that had previously been blocked with 1% BSA. The tubes were centrifuged at 14,000 rpm for 3 min and the supernatant was aspirated. The cell pellets were washed two times with cold serum-free medium without resuspension of the pellet. The pellet was then lysed and dissolved in SDS–polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer without β-mercaptoethanol and boiled for 5 min. The proteins were resolved by SDS-PAGE and protein immunoblotting with the 9B antibody.
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18. Expression of a *KuzDN* construct lacking the proprotein and metalloprotease domains was driven by a GAL4 line 32B.
19. More than 2400 lethal P-element insertions were screened for phenotypic modification effects on *KuzDN*. Seven P-element insertions reduced viability of the *KuzDN* flies (were semi-lethal) when the flies were also heterozygous for each of the P insertions. Preliminary characterization of these P insertions revealed that two of them were *kuz* alleles and one was a loss-of-function *delta* allele; the nature of the other insertions is unknown.
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21. The IgG fraction of 9B ascites fluid was purified with a HiTrap recombinant protein A–Sepharose (Pharmacia) and subsequently coupled to CNBr-activated Sepharose (Pharmacia). *Drosophila* Dl-S2 cells were induced with 0.7 mM $CuSO_4$ in serum-free medium for 2 days, and the medium was collected and precipitated with 70% ammonium sulfate. The precipitate was collected by centrifugation and subsequently resuspended and dialyzed against 20 mM Hepes, 150 mM NaCl, and 2 mM $CaCl_2$ (pH 7.4). This sample was passed over the 9B-Sepharose, washed with 1.0 M NaCl, and eluted with 25 mM glycine, pH 2.8, and immediately neutralized with 1M tris-HCl. NH_2-terminal amino acid analysis was performed with an Applied Biosystems gas phase sequencer in the Keck Foundation Biotechnology Resource Laboratory, Yale University.
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23. The *KuzDN* construct used for the genetic screen and full-length *kuz* were cloned into pCaSpeR3 for expression under the heat shock promoter. Cotransfections with these constructs and those of Pan *et al.* (*6*) (Fig. 3, A and B) gave identical results.
24. Expression of N and Dl in S2 cells was induced for 16 hours with 0.085 mM and 0.022 mM $CuSO_4$, respectively. The cells were then centrifuged and raised in serum-free medium to an equivalent density yielding between 20 and 30% transmitted light at 320 nm (T_{320nm}) (~2×10^6 cells per milliliter) in a Benchtop spectrophotometer. Blank values were set with M3 medium alone. Four hundred microliters of N cells and 400 μl of Dl cells were then pipetted into a 1.4-ml, black-sided, stoppered quartz cuvette that was then quickly inverted three times. The T_{320nm} was read immediately to determine the time "zero" value. The cuvette was then rocked horizontally on a Thermolyne vari-mixer at 20 oscillations per minute, and subsequent T_{320nm} readings were taken at 1-min intervals. Changes in T_{320nm} (relative to time zero) were then plotted versus time. The effect of Dl^{EC} was compared to a control concentrate of medium from S2 cells stably transfected with an irrelevant construct [ΔECN (*12*)]. N-S2 cells (400 μl) were preincubated with either 5× Dl^{EC} or 5× ΔECN (100 μl) for 10 to 60 min and subsequently aggregated with Dl-S2 cells as described above.

25. The authors thank D. Pan and G. Rubin for providing *kuz* and *kuzDN* constructs, R. Mann for his help at many different levels, and M. Rudnick for technical assistance. Supported by the Pew Scholars Program in the Biomedical Sciences and the Lucille P. Markey Charitable Trust (T.X.) and from NIH grants NS14841 (P.R.) and NS26084 (S.A.T.).

14 July 1998; accepted 26 October 1998

Plant Paralog to Viral Movement Protein That Potentiates Transport of mRNA into the Phloem

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CmPP16 from *Cucurbita maxima* was cloned and the protein was shown to possess properties similar to those of viral movement proteins. *CmPP16* messenger RNA (mRNA) is present in phloem tissue, whereas protein appears confined to sieve elements (SE). Microinjection and grafting studies revealed that CmPP16 moves from cell to cell, mediates the transport of sense and antisense RNA, and moves together with its mRNA into the SE of scion tissue. CmPP16 possesses the characteristics that are likely required to mediate RNA delivery into the long-distance translocation stream. Thus, RNA may move within the phloem as a component of a plant information superhighway.

Phloem represents an advanced long-distance transport system that delivers nutrients and hormones to plant tissues and organs. Mature SE are enucleate (*1*) and thus must rely on their associated companion cells (CC) for maintenance of their physiological functions (*2*). To this end, SE are connected to CC through specialized, branched plasmodesmata (*3*) that mediate delivery of proteins into the long-distance translocation stream (*4, 5*).

The observation that specific mRNA molecules, such as sucrose transporter 1 (*SUT1*)

RNA, are present within CC-SE plasmodesmata and in parietal locations in functional SE (*6*) suggests that RNA can similarly traffic through CC-SE plasmodesmata. These findings are consistent with experiments that probe the mechanism by which plant viruses establish a systemic infection. Genetic, molecular, and cellular approaches have established that plant viruses express movement proteins (MP) having the capacity to interact with plasmodesmata to mediate cell-to-cell transport of MP and viral nucleic acid–MP complexes (*7–9*). Thus, plant viruses likely have evolved the capacity to exploit the endogenous pathways utilized by the plant to traffic macromolecules from their sites of synthesis into surrounding cells (*1*).

Delivery of RNA to distant tissues and developing organs may reflect a mechanism used by plants to regulate translational events (*11*). Operation of this endogenous RNA translocation system could involve phloem-specific proteins whose functions would parallel those of plant viral MPs. To identify such proteins within the phloem sap (Fig. 1A), we used polyclonal antibodies raised against the 35-kD MP of red clover necrotic mosaic virus (RCNMV) (*12*) in immunoblot analyses (Fig. 1B). Here we report on the isolation and characterization of the 16-kD *Cucurbita maxima* (pumpkin) phloem protein (CmPP16) that displays functional similarity, and a limited degree of sequence identity, to the MP of RCNMV.

CmPP16 was purified, its NH_2-terminus was sequenced, and reverse transcriptase–polymerase chain reaction (RT-PCR) was used to amplify a *CmPP16* probe (*13*). We identified two cDNA clones from a pumpkin stem cDNA library constructed from stem mRNA (*14*) that encode proteins with predicted masses of 16.5 and 15.6 kD. These cDNA clones shared 82% identity at the amino acid level and were designated *CmPP16-1* and *CmPP16-2*, respectively. We synthesized a fragment of CmPP16 by PCR and identified four introns within the *CmPP16-1* genomic clone (Fig. 2A). This result established that these are endogenous plant genes, not derived from viral RNA, which would lack introns (and could have been present in the phloem of infected plants). Hybridization of ^{32}P-labeled *CmPP16-1* RNA to a genomic Southern blot identified two genes (*15*). Recombinant His_6-CmPP16-1 (R-CmPP16-1) was produced in *Escherichia coli* and used to generate polyclonal antiserum. Immunoblot analysis with this R-CmPP16-1 antiserum recognized R-CmPP16-1, endogenous CmPP16, and the RCNMV MP (Fig. 1C). A BLAST search identified homologues in rice and maize (Fig. 2B);

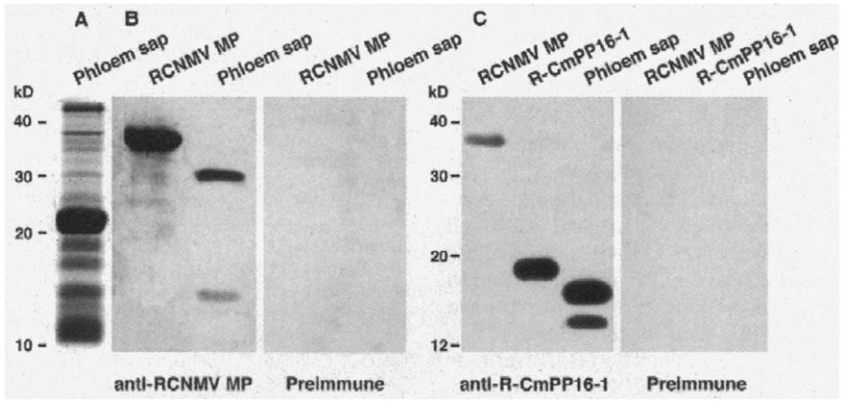

Fig. 1. Immunodetection of pumpkin phloem proteins. Phloem proteins were extracted (*4*), resolved by SDS–polyacrylamide gel electrophoresis (SDS-PAGE), and either stained with Coomassie blue (**A**) or immunoblotted (*14, 24*) to identify proteins that cross-react with antiserum to RCNMV MP (**B**) or polyclonal antibodies to R-CmPP16-1 (**C**). Samples tested include recombinant RCNMV MP, recombinant CmPP16-1, and phloem sap proteins (*4*). Because R-CmPP16-1 contains a histidine tag and a peptide linker, it is about 2 kD larger than the endogenous proteins, which differ in mass by 1 kD, in agreement with the deduced size of the CmPP16-1 and CmPP16-2 proteins (Fig. 2). We did not detect a reaction with either antiserum directed against a total protein preparation extracted from *E. coli* or with preimmune serum. Total phloem protein, 50 μg per lane; recombinant proteins (RCNMV MP and R-CmPP16-1), 5 μg per lane.

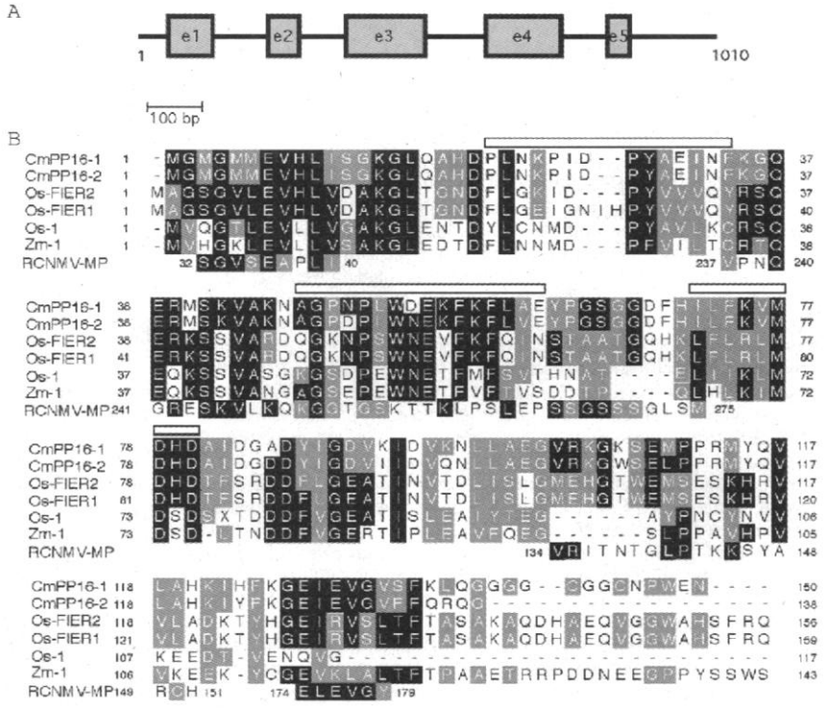

Fig. 2. Gene structure and comparison of CmPP16 and related proteins. (**A**) Schematic representation of *CmPP16-1* gene displaying its five exons. (**B**) The *CmPP16-1* (GenBank accession number AF079170) and *CmPP16-2* (GenBank accession number AF079171) genes encode proteins of 16.5 and 15.6 kD, respectively (*23*). Homologous proteins were identified in rice (Os-FIERG2, accession no. U95136; Os-FIERG1, accession no. U95135; Os-1, accession no. D24373) and maize (Zm-1, accession no. U64437); the regions of greatest identity were present toward the NH_2-terminus. A PKC-like C_2 Ca^{2+}/phospholipid-binding domain (*16*) is indicated by open rectangles. A comparison of blocks (*25*) of these proteins with RCNMV MP identified four domains having a high antigenic index (DNAstar, Madison, Wisconsin). Black boxes indicate identity, and shaded boxes indicate conservative changes. MegAlign software was used to align sequences (*25*).

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incomplete sequences for two additional genes were also present in the *Arabidopsis* expressed sequence tag database.

Immunocytochemical experiments established that CmPP16 protein is confined to the SE periphery within the vascular tissue (Fig. 3, C and D; schematic transverse section of pumpkin stem), which suggests that it may be associated with the plasma membrane. Consistent with this observation, we found that CmPP16 contains a C_2 domain (Fig. 2B) present in the Ca^{2+}-binding protein kinase C (PKC) family (*16*). Northern blot (Fig. 3B) and immunocytochemical localization analyses demonstrated that *CmPP16* mRNA and protein are present in the SE of leaves, stems, roots, and flowers. Finally, both high-resolution in situ hybridization and in situ RT-PCR (*17*) methods demonstrated that the *CmPP16* mRNA is predominantly located within CC, and to a lesser extent in SE, of the functional phloem of petiole and stem tissues (Fig. 3, E to H).

Sequence comparison of CmPP16 and RCNMV MP showed four motifs that display similarity (Fig. 2B) and likely account for the amount of immunological cross-reactivity detected between these proteins. Gel mobility-shift assays were next performed to test the capacity of these proteins to interact with RNA. R-CmPP16-1 was able to bind both sense and antisense *CmPP16-1* RNA as well as RCNMV RNA2 (Fig. 4A). An equivalent capacity for RNA binding was displayed by the RCNMV MP (Fig. 4B). Similar to RCNMV MP (*8*), R-CmPP16-1 bound RNA but failed to interact with both single-stranded and double-stranded DNA (Fig. 4, D and E,

Table 1. R-CmPP16-1 interacts with mesophyll plasmodesmata to mediate its own cell-to-cell transport and potentiate the trafficking of sense and antisense RNA. Lucifer yellow CH and 11-kD FITC-dextran were from Molecular Probes. TOTO, TOTO-1 iodide; TRITC, tetramethylrhodamine-5-(and 6)-isothiocyanate.

Injected material	Microinjection	
	Total (*n*)	Movement* [*n* (%)]
Lucifer yellow CH	32	31 (97)
FITC-dextran (11 kD)	40	1 (2.5)
R-CmPP16-1 + 11-kD FITC-dextran	25	21 (84)
Endogenous CmPP16 + 11-kD FITC-dextran	60	48 (80)
TRITC–R-CmPP16-1	21	17 (80)
TRITC–R-CmPP16-1 + sense CmPP16-1 RNA-CF	20	15 (75)
TRITC–R-CmPP16-1 + antisense CmPP16-1 RNA-CF	20	15 (75)
TRITC–R-CmPP16–1 + RCNMV sense RNA 2-CF	10	8 (80)
CmPP16-1 ss-DNA-TOTO	10	0 (0)
TRITC–R-CmPP16-1 + CmPP16-1 ss-DNA-TOTO	10	0 (0)
TRITC–RCNMV MP	10	8 (80)
TRITC–RCNMV MP + CmPP16-1 sense RNA-CF	10	8 (80)
CmPP16-1 sense RNA-CF	20	3 (15)
CmPP16-1 antisense RNA-CF	20	2 (10)
RCNMV sense RNA 2-CF	10	1 (10)
SUT1 sense RNA-CF†	10	1 (10)
SUT1 RNA-CF + TRITC–R-CmPP16-1	10	10 (100)

*Number of injections and percent of total injections in which the probe moved into surrounding mesophyll cells. In coinjection experiments, the distribution patterns of TRITC-labeled protein and CF-labeled RNA were coincident, as deduced from simultaneous multiple-channel fluorescence analysis performed with a CLSM [see supplementary material (Fig. 3) at www.sciencemag.org/feature/data/982968.shl]. †SUT1 from potato (*6*) was CF labeled as described in (*18*).

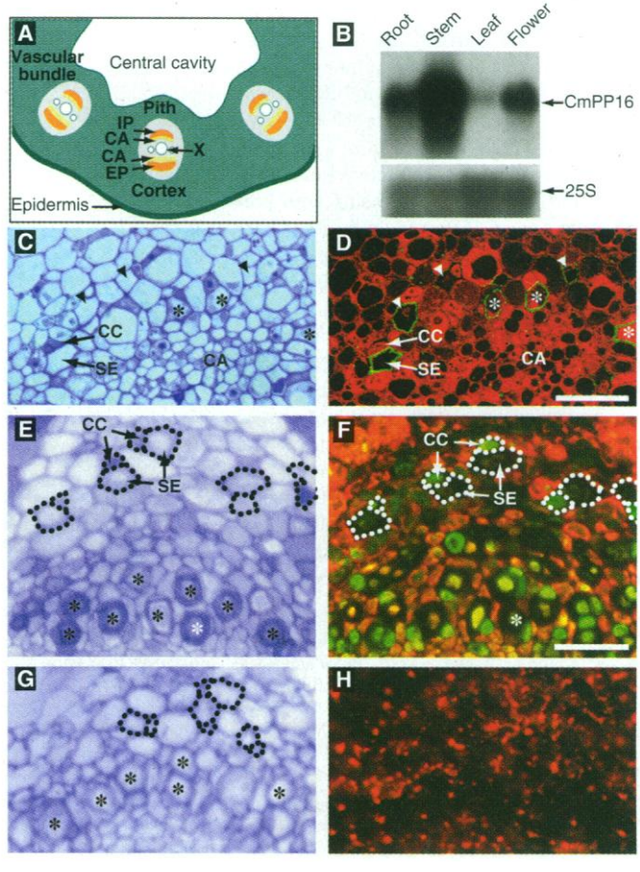

Fig. 3. Localization of *CmPP16* mRNA and protein in *C. maxima* vascular tissue. (**A**) Schematic transverse section of a pumpkin stem. Vascular bundles are composed of internal and external phloem (IP and EP, respectively), cambium (CA), and xylem (X) [see supplementary material (Fig. 2) at www.sciencemag.org/feature/data/982968.shl]. Such bundles are distributed in a ring around the outer region of the stem (or petiole). (**B**) Northern blot analyses establish that the *CmPP16* RNA is located in a variety of plant tissues. Total RNA (10 μg) from the indicated tissue was electrophoresed, blotted (*14*), probed with ^{32}P-labeled CmPP16 open reading frame at 65°C, and washed with 0.1× SSC (0.015 M NaCl and 0.0015 M sodium citrate). (**C**) Cellular arrangement within the IP illustrating functional (arrowheads) and immature (asterisks) SE derived from the CA. Semithin section stained with toluidine blue. (**D**) Confocal laser scanning microscope (CLSM) image of a pumpkin semiserial section [see (C)] labeled with antiserum to R-CmPP16-1 reveals the presence of CmPP16 within the phloem (*26*). CmPP16 signal (green fluorescence) is present at the periphery of mature and immature SE. Tissue structure was observed (red fluorescence) with Safranin O used as a histochemical stain. Controls with preimmune sera yielded images devoid of fluorescent signal. (**E** to **H**) CLSM images of transverse pumpkin petiole sections processed for in situ RT-PCR (*17*). To identify cellular structure, we collected images (E and G) before removal of unincorporated CF-labeled deoxyuridine triphosphate. Asterisks identify immature SE. *CmPP16* mRNA is detected (green fluorescent signal) within immature CC-SE complexes and, predominantly, in mature CC (F); note low but detectable amount of green fluorescent signal in SE. White asterisks in (E) and (F) facilitate identification of the same cell in the two images. In control experiments, tissues were treated in the same manner except that primers were omitted (H). Red fluorescence in (F) and (H) represents autofluorescence. Scale bars, 50 μm; bar in (D) is common to (C) and bar in (F) is common to (E), (G), and (H).

respectively). Bovine serum albumin was used as a control, showing no interaction with DNA (Fig. 4C). To examine the functions of these two proteins, we carried out a series of microinjection experiments (*18*). As found for the RCNMV MP (*8*), R-CmPP16-1 has the capacity to interact with plasmodesmata to induce an increase in size-exclusion limit, potentiate its own cell-to-cell transport, and mediate the trafficking of RNA (Table 1).

Consistent with our gel mobility-shift assays, R-CmPP16-1 mediated the cell-to-cell transport of both sense and antisense RNA of two different sequences but was unable to effect the movement of single- or double-stranded DNA. No cell-to-cell movement was detected when chromatide fluorescein (CF)-labeled RNA or DNA-TOTO (Molecular Probes) alone was introduced into a target mesophyll cell (Table 1). These results support the hypothesis that R-CmPP16-1 mediates the cell-to-cell transport of RNA through mesophyll plasmodesmata (*19*).

Grafting experiments between pumpkin and cucumber plants provided another test of the capacity of endogenous CmPP16 to move from the CC into the SE through plasmodesmata. Phloem sap was collected from the cucumber scion of 10-day-old grafted plants (*5*), and proteins were electrophoresed, blotted, and immunodetected with antiserum to R-CmPP16-1. Endogenous CmPP16 was present in the phloem sap of both pumpkin and the cucumber scion (Fig. 5A). Hence, the heterograft, immunolocalization, and microinjection experiments establish that CmPP16 protein has the capacity to move from the CC into the phloem long-distance translocation stream. Aliquots of these same phloem saps were used to extract RNA, which was then amplified by RT-PCR, which revealed the presence of *CmPP16-1* mRNA in both pumpkin (control) and the cucumber scion but not in homografted cucumber (Fig. 5B). These results are consistent with our in situ RT-PCR, gel mobility-shift, and microinjection assays and provide support for the hypothesis that CmPP16 protein plays a role in mRNA delivery into the phloem translocation stream.

Our results add to the emerging picture of non–cell-autonomous regulation of gene expression in plants (*20*). The discovery that sequence-specific cosuppression can operate through an imposed graft union (*21*) likely reflects the involvement of RNA transport through the phloem (*11*). The ability of R-CmPP16-1 to mediate cell-to-cell transport of both sense and antisense transcripts of various sequences provides a possible molecular basis to explain how plants can translocate RNA present within the functional sieve tube system (*17*).

Insight into the possible involvement of supracellular control over developmental processes in animal systems has come from studies in which double-stranded RNA was microinjected into *Caenorhabditis elegans* (*22*). It will be intriguing to learn the extent to which the macromolecular trafficking systems used by plants and animals share common features.

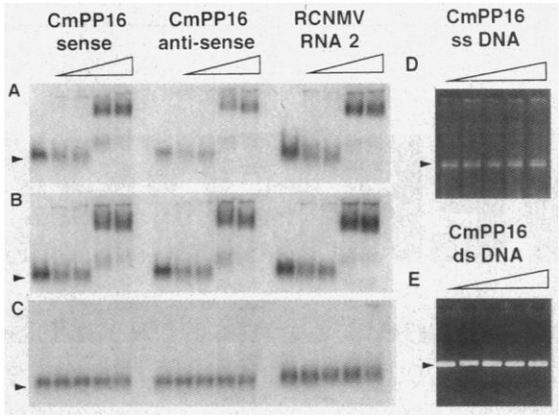

Fig. 4. R-CmPP16-1 binds various RNA molecules. Electromobility-shift assays were performed by mixing R-CmPP16-1 (**A**) or RCNMV MP (**B**) with 50 ng of the appropriate ^{32}P-labeled RNA in binding buffer (*8*). These mixtures were incubated for 1 hour at 4°C, resolved in 1% (w/v) agarose gels, and then processed for autoradiography. (**C**) Control RNA binding assays were performed with bovine serum albumin. The ^{32}P-labeled RNA probe was synthesized in vitro (MAXIscript kit, Ambion, Austin, Texas). A similar series of electromobility-shift assays was performed with single-stranded (ss) (**D**) and double-stranded (ds) (**E**) DNA. DNA was electrophoresed as in (A) and visualized by ethidium bromide staining. The amounts of protein used in each experiment were as follows: 0.0, 0.1, 0.2, 0.4, and 0.8 μg. Both R-CmPP16-1 and RCNMV MP interact with RNA in a manner consistent with cooperative binding. However, R-CmPP16-1 did not interact with DNA. Both R-CmPP16-1 and RCNMV MP formed complexes with sense and antisense RNA, and similar threshold amounts of protein were required for RNA retardation. In contrast, bovine serum albumin failed to retard the RNA probes.

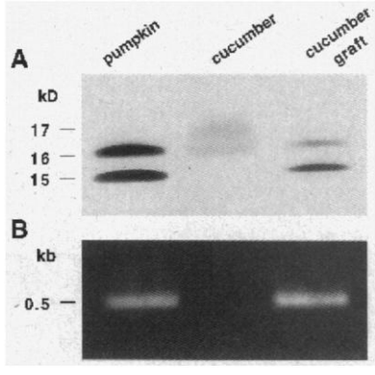

Fig. 5. Endogenous CmPP16 and its RNA move in the phloem from pumpkin stock into a heterografted scion. Phloem sap was collected from pumpkin, cucumber, and cucumber scion heterografted onto pumpkin stock (*5*). (**A**) Proteins were resolved in a 4 to 20% SDS-PAGE gradient and then immunodetected with antiserum to R-CmPP16-1. CmPP16-1 and CmPP16-2 are present in phloem sap collected from pumpkin (control) and the heterografted cucumber scions, indicating that the two forms of CmPP16 move within the phloem long-distance translocation stream, albeit with seemingly different efficiencies. Two putative CmPP16 homologues are present in the phloem sap collected from cucumber but are absent or present at much reduced amounts in the sap of heterografted cucumber scions. Absence of the cucumber homologues from the scion phloem sap may reflect a block on their entry into the SE by the pumpkin proteins. Total phloem protein loaded was 50 μg per lane. (**B**) *CmPP16* RNA is present in phloem sap collected from pumpkin and heterografted cucumber scion but absent from sap obtained from cucumber. *CmPP16* RNA was analyzed by RT-PCR (*17*).

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13. CmPP16 was purified as described in (*4*) and blotted to polyvinylidene difluoride membranes for microsequencing. The sequence GMGMMEVHLISXGKGLQAHD (*23*) was obtained from the NH_2-terminus. To clone CmPP16 by 3′ rapid amplification of cDNA ends (RACE)-PCR, a degenerate primer was designed—16A1 [5′-ATGGGIATGATGGARGTICA-3′ (where R is A or G)]—and oligo(dT) was used as the reverse primer. A pumpkin cDNA library was prepared from stem tissue excised from 2-month-old plants (*17*). Pumpkin stem cDNA was synthesized (Lambda ZAP II cDNA synthesis kit, Stratagene, La Jolla, CA) and used as a template for PCR. The first amplification was done with primer 16A1 and oligo(dT) under the following conditions: 1 min at 94°C, 1 min at 59°C, 2 min at 72°C (30 cycles). The resultant PCR fragments

were subcloned into the pCR2.1 vector (Invitrogen, Carlsbad, CA) and sequenced. The clone corresponding to CmPP16 was used to screen the stem cDNA library.
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17. We used the side-grafting technique to graft the apical 10-cm region of 4-week-old cucumber plants onto 4-week-old pumpkin plants (*5*). Ten days later, we severed these scions (cut was 3 cm from graft union) and collected phloem sap from the basal end (*4*). We also collected phloem sap from the equivalent position on control cucumber plants. Aliquots (5 μl) of sap were mixed with an equal volume of 8 M guanidinium HCl and extracted twice with a 25:24:1 mixture of phenol/chloroform/isoamyl alcohol [J. Logemann, J. Schell, L. Willmitzer, *Anal. Biochem.* **163**, 16 (1987)]. RNA was precipitated, centrifuged at 4°C for 45 min, and then resuspended in deionized sterile water. We isolated polyadenylated RNA from total RNA by using an oligotex mRNA extraction kit (Qiagen) and synthesized first-strand cDNA with Superscript reverse transcriptase (Gibco). We then used this cDNA as template for long-distance PCR (Clontech) according to the manufacturer's recommendations with a Robocycler PCR system (Stratagene). We used specific primers to amplify the *CmPP16* cDNA according to the following protocol: 1 min at 94°C (one cycle); 30 s at 94°C, 30 s at 61°C, 70 s at 72°C (35 cycles). Preliminary RT-PCR experiments conducted on pumpkin and cucumber confirmed the specificity of the primers designed to amplify *CmPP16*. The amplified products were run on an agarose gel and visualized directly by ethidium bromide staining.
18. Preparation of fluorescent probes and microinjection procedures are described in (*9, 10*).
19. Preliminary experiments in which the biolistic bombardment method [A. Itaya *et al., Plant J.* **12**, 1223 (1997)] was used to deliver various combinations of fluorescein isothiocyanate (FITC)–labeled R-CmPP16-1 and RCNMV FITC-MP with or without CF-labeled RNA yielded results consistent with our microinjection experiments.
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23. Abbreviations for amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E. Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
24. Polyclonal antibodies directed against recombinant CmPP16-1 and RCNMV MP were raised in rabbits; preimmune serum was tested against the antigens before the immunological scheme. Immunoglobulin G–purified antibody preparations were used in immunoblot analyses.
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26. Semithin sections (500 to 750 nm) of pumpkin stems and petioles were prepared and processed for CmPP16 immunolocalization by the procedures described by Wang *et al.* [H.-L. Wang, Y. Wang, D. Giesman-Cookmeyer, S. A. Lommel, W. J. Lucas, *Virology* **245**, 75 (1998)].
27. R.R.-M. was partially supported by a postdoctoral fellowship from CONACYT-México. Supported by Department of Energy Biosciences grant DE-FG03-94ER20134 and National Science Foundation grant IBN-94-06974 (W.J.L.). We thank the many colleagues whose comments and advice contributed to the development of this work. The RCNMV MP antibody used in initial screening studies was generously provided by S. Lommel, North Carolina State University. The plasmid containing the potato *SUT1* was kindly provided by W. Frommer, University of Tübingen, Germany.

3 June 1998; accepted 19 November 1998