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Garlic virus X 11-kDa protein granules move within the cytoplasm and traffic a host protein normally found in the nucleolus

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SUMMARY

The subcellular localization of the 11-kDa protein (p11) encoded by ORF3 of *Garlic virus X* (GarVX; genus *Allexivirus*, family *Alphaflexiviridae*) was examined by confocal microscopy. Granules with intense fluorescence were visible on the endoplasmic reticulum when p11 fused with green or red fluorescent protein (GFP or RFP) was expressed in epidermal cells of *Nicotiana benthamiana*. Moreover, the p11-RFP granules moved in the cytoplasm, along the cell periphery and through the cell membranes to adjacent cells. A 17-kDa protein (p17) of garlic interacting with p11 was identified by yeast two-hybridization and bimolecular fluorescence complementation assay. When p17 fused to GFP was expressed in epidermal cells of *N. benthamiana*, it localized to the nucleolus. However, in the presence of GarVX p11, the distribution of p17 changed to that of p11, but did not appear to affect the pattern of movement of p11.

INTRODUCTION

Garlic virus X (GarVX) is one of several members of the genus *Allexivirus* (family *Alphaflexiviridae*), all of which have been detected only in *Allium* spp. and are thought to be transmitted by mites (Adams *et al*., 2004; Kang *et al*., 2007; Song *et al*., 1997). The genomic organization is similar throughout the genus (Chen *et al*., 2001; Kanyuka *et al*., 1992; Song *et al*., 1998; Sumi *et al*., 1999) and resembles that of carlaviruses, potexviruses and foveaviruses, with a single-stranded polyadenylated RNA genome encoding a large α-like replicase and four to five smaller open reading frames (ORFs) (Chen *et al*., 2004; Song *et al*., 1998; Sumi *et al*., 1999). In carlaviruses, potexviruses and foveaviruses, the partially overlapping ORFs 2–4 constitute the triple gene block (namely TGB1, TGB2 and TGB3), the proteins of which are necessary for cell-to-cell movement. ORF5 encodes the coat protein. In carlaviruses, ORF6 encodes a nucleic acid-binding protein that is not present in potexviruses and foveaviruses. Allexiviruses are similar to carlaviruses in organization, but the third TGB ORF lacks a classical initiation codon and may not encode a protein. The following ORF (ORF4) encodes a 40-kDa serine-rich protein with no known homology to other reported proteins (Chen *et al*., 2004; Song *et al*., 1998).

Although allexiviruses have been characterized and molecular techniques for their detection developed (Chen *et al*., 2001, 2004; Song *et al*., 1997; Sumi *et al*., 1999), gene function has only been inferred from studies of carlaviruses, potexviruses and foveaviruses. Here, we report experiments on the subcellular localization of the GarVX 11-kDa protein encoded by ORF3 (presumed to be TGB2, p11) and the identification of a nucleolar protein interacting with it in garlic (*Allium sativum* L.).

RESULTS

Subcellular localization of p11

p11 of GarVX has low amino acid identity to TGB2 proteins of carlaviruses, potexviruses and foveaviruses (Table S1, see Supporting Information). However, they all contain two transmembrane (TM) segments (Fig. S1, see Supporting Information). Experimental studies have shown that green fluorescent protein (GFP)-fused TGB2 proteins are associated with the endoplasmic reticulum (ER) (Ju *et al*., 2005; Samuels *et al*., 2007). To probe the location of p11 in plant cells, GFP was fused to its C-terminus (p11-GFP). As a control, partial GUS protein (ΔGUS) containing

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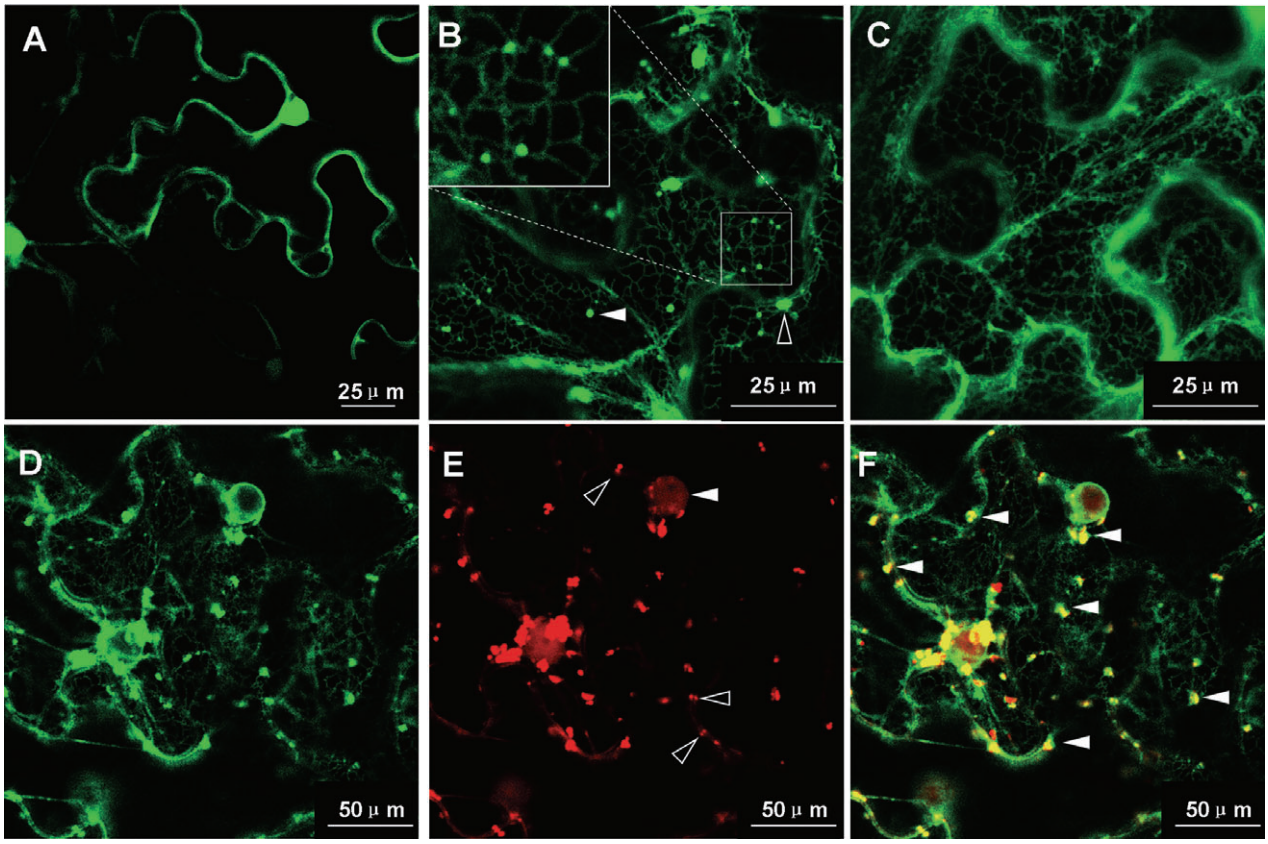

Fig. 1 Localization of p11 in *Nicotiana benthamiana* and 16c epidermal cells. (A) Localization of pΔGUS-GFP (GFP, green fluorescent protein) in *N. benthamiana* (control). (B) Localization of p11-GFP in *N. benthamiana*. The enlarged zone reveals that the fluorescence forms a network and that the green fluorescent granules exist on it. The network is probably the endoplasmic reticulum (ER) with the results shown in (C)–(F). The white triangle points to a granule. (C) Image showing fluorescence of 16c epidermal cell with fluorescence evenly distributed on ER. (D) Image showing green fluorescence when p11-RFP (RFP, red fluorescent protein) was expressed in 16c. Vesicles of different sizes were formed on ER. (E) Image showing red fluorescence when p11-RFP was expressed in 16c. p11-RFP formed granules in the cytoplasm and at the periphery of the cells. Black triangles point to the twin structures formed by p11-RFP granules on the cell periphery. The white triangle points to the nucleus. (F) Merged image of (D) and (E). White triangles point to the overlapped p11-RFP granules and ER-derived vesicles.

equal amino acid numbers to those of p11 was fused to GFP (pΔGUS-GFP). Both constructs were cloned behind the 35S promoter of pCV1300 to create pCV:p11-GFP and pCV:pΔGUS-GFP. These were expressed in leaf cells of *Nicotiana benthamiana* and, 3 days post-infiltration (dpi), green fluorescence was monitored by confocal microscopy. In cells expressing pCV:pΔGUS-GFP, fluorescence was cytoplasmic and nuclear (Fig. 1A), but fluorescence was seen along ER and there were fluorescent granules formed on ER in cells expressing pCV:p11-GFP (Fig. 1B), indicating that p11-GFP was associated with ER.

To further demonstrate that p11-GFP associates with ER, pCV:p11-RFP (RFP, red fluorescent protein) was constructed and transiently expressed in leaves of transgenic *N. benthamiana* 16c line. This line expresses ER-binding GFP which is distributed on ER and the cell periphery (Fig. 1C), but, when p11-RFP was expressed in the cells, vesicles with different sizes were formed on ER and the cell periphery (Fig. 1D). Under the red channel for the detection of the localization of p11-RFP, it was revealed that p11-RFP formed granules in the cytoplasm and at the periphery of cells (Fig. 1E). When merged with the image showing green fluorescence, nearly all of the p11-RFP granules overlapped with ER-derived vesicles (Fig. 1F). These results confirm that p11-RFP is associated with ER. It was noticeable that the p11-RFP granules were also formed on the cell periphery and were sometimes twin structures along the walls of adjacent cells (Fig. 1E). They were presumed to be the peripheral bodies reported to occur in cells infected by several viruses (Solovyev *et al*., 2000; Zamyatnin *et al*., 2002).

In addition to the p11-RFP granules on ER and at the cell periphery, there was also red fluorescence in the nucleus (Fig. 1E). As the molecular weight of RFP-fused p11 was approximately 38 kDa, smaller than the maximum size of protein that can diffuse through nuclear pores into the nucleus (Wang and

Brattain, 2007), we could not eliminate the possibility that p11-RFP had diffused into the nucleus.

Movement of p11-RFP granules

In confocal microscopy, the p11-RFP granules moved in the cytoplasm and along the cell periphery. To record the movement of p11-RFP granules, four sequential photographs were taken over a period of 30 s (Fig. 2). Images detecting green fluorescence and red fluorescence were taken simultaneously at 0, 10, 20 and 30 s, and then merged to record the location of green vesicles which co-localized with p11-RFP granules. In addition, a video recording the movement of p11-RFP under the red channel was taken (Video S1, see Supporting Information). The granules appeared to move along the long ER strands that spanned both cell and cortical ER, as seen most clearly under the green channel (Video S2, see Supporting Information) (Fig. 2). The granules moved at different speeds and often moved into another focal plane and disappeared from the visual field (Videos S1 and S2).

p11-RFP formed twin structures along the walls of adjacent cells and moved in cells, which prompted us to investigate whether it moved from cell to cell. Cell-to-cell movement of viral proteins is usually mediated by plasmodesmata (PD). To demonstrate the localization of p11 in relation to PD, we used GFP-fused *Tobacco mosaic virus*-movement protein (TMV-MP) as a PD marker. The results showed that, when GFP-fused TMV-MP and RFP-fused p11 were co-expressed in epidermal cells of *N. benthamiana*, most RFP-fused p11 on the cell periphery co-localized with TMV-MP (Fig. 3A). In addition, when the epidermal cells co-expressing both proteins were plasmolysed, the p11-RFP remaining on the cell wall also co-localized with TMV-MP-GFP (Fig. 3B), and there were p11-RFP particles localizing on the strands between PD and the cell membrane (Fig. 3C). These observations strongly suggest that p11-RFP can move between cells through PD.

A fluorescence diffusion experiment was then designed to demonstrate the ability of p11 to move from cell to cell. A 1-μL sample of *Agrobacterium* containing the pCV:p11-GFP construct was dropped on a tiny hole punched by a needle in an *N. benthamiana* leaf. The epidermal cells of the first layer around the hole were infected by *Agrobacterium* and thus expressed p11-GFP transiently. Three days after inoculation with *Agrobacterium* containing pCV:pΔGUS-GFP (control), fluorescence was restricted to the first layer of cells around the hole (Fig. 4A). However, when *Agrobacterium* containing pCV:p11-GFP was used, the intensity of fluorescence was less, but was visible in more distant cells (two to four cell lengths; Fig. 4B), confirming the ability of GFP-fused p11 to move from cell to cell. This was further confirmed by the expression of pCV:p11-GFP or pCV:pΔGUS-GFP in *N. benthamiana* leaves by bombardment. Fluorescence was restricted to single cells expressing pCV:pΔGUS-GFP (Fig. 4C, Table 1) but, in the pCV:p11-GFP treatment, fluorescence was visible in both the initial cell and its neighbours in 59% of observations (Fig. 4D, Table 1).

A garlic p17 protein interacts with p11

A yeast two-hybridization screen was used to identify garlic proteins potentially interacting with p11. Using p11 as bait, we identified one yeast clone growing on stringent Leu–, Trp–, His– and Ade– medium, which produced blue positive results in α-galactosidase (X-α-Gal) assays (data not shown). Sequencing analysis showed that the sequence in this clone contained a whole ORF with 459 nucleotides, together with a 5′-untranslated region (5′-UTR) of 53 nucleotides and a 3′-UTR of 108 nucleotides, before the polyA (Accession Number: HM326891; Fig. 5). The ORF was predicted to encode a 17-kDa protein (p17) with 152 amino acids (Fig. 5).

To confirm this interaction in plant cells, we cloned p17 and p11 into pCV-nYFP-C and pCV-cYFP-C constructs (YFP, yellow fluorescent protein), respectively, forming pCV-p17-nYFP and pCV-p11-cYFP for bimolecular fluorescence complementation (BiFC) assay. Both BiFC constructs were expressed in *N. benthamiana* epidermal cells by *Agrobacterium* infiltration. YFP fluorescence (recorded as green during imaging) was observed at 3 dpi in the cells co-expressing p17-nYFP and pCV-p11-cYFP (Fig. 6A), but not in the control cells co-expressing p17-nYFP and pΔGUS-cYFP, or pΔGUS-nYFP and p11-cYFP (data not shown), suggesting that there was an interaction between the viral p11 and the garlic p17. Moreover, YFP fluorescence spots were localized in the cytoplasm and nucleus, but not in the nucleolus.

The garlic p17 protein is a nucleolar protein

No sequences very closely related to that of garlic p17 could be identified by BLASTN, but several plant proteins of unknown function share two conserved regions, (L/I)RD(A/S)YV and KEYDEK(M/V)(L/I)(V/I)EQ(I/Y)(R/K), suggesting that they may be related in function (Fig. S2, see Supporting Information). The proteins are from *Arabidopsis thaliana* (NP_191824), *Oryza sativa* (NP_001042850), *Picea sitchensis* (ABK21989), *Populus trichocarpa* (XP_002302741), *Ricinus communis* (XP_002515331), *Sorghum bicolor* (XP_002457739), *Vitis vinifera* (XP_002274279) and *Zea mays* (NP_001159124). Motif scan analysis showed that these proteins, except that from *Sorghum bicolor*, contained a potential nuclear localization signal (NLS) at their N-termini (Figs 5, S2), suggesting that they are nuclear proteins. The localization of garlic p17 was therefore examined by expressing a construct with GFP fused to its C-terminus in *N. benthamiana* epidermal cells. GFP fluorescence was detected from a distinct spot within the nucleus, but not from the cytoplasm or cellular membrane system (Fig. 6B,C). When RFP-fused fibrillarin (Fib) (a known nucleolar protein) was

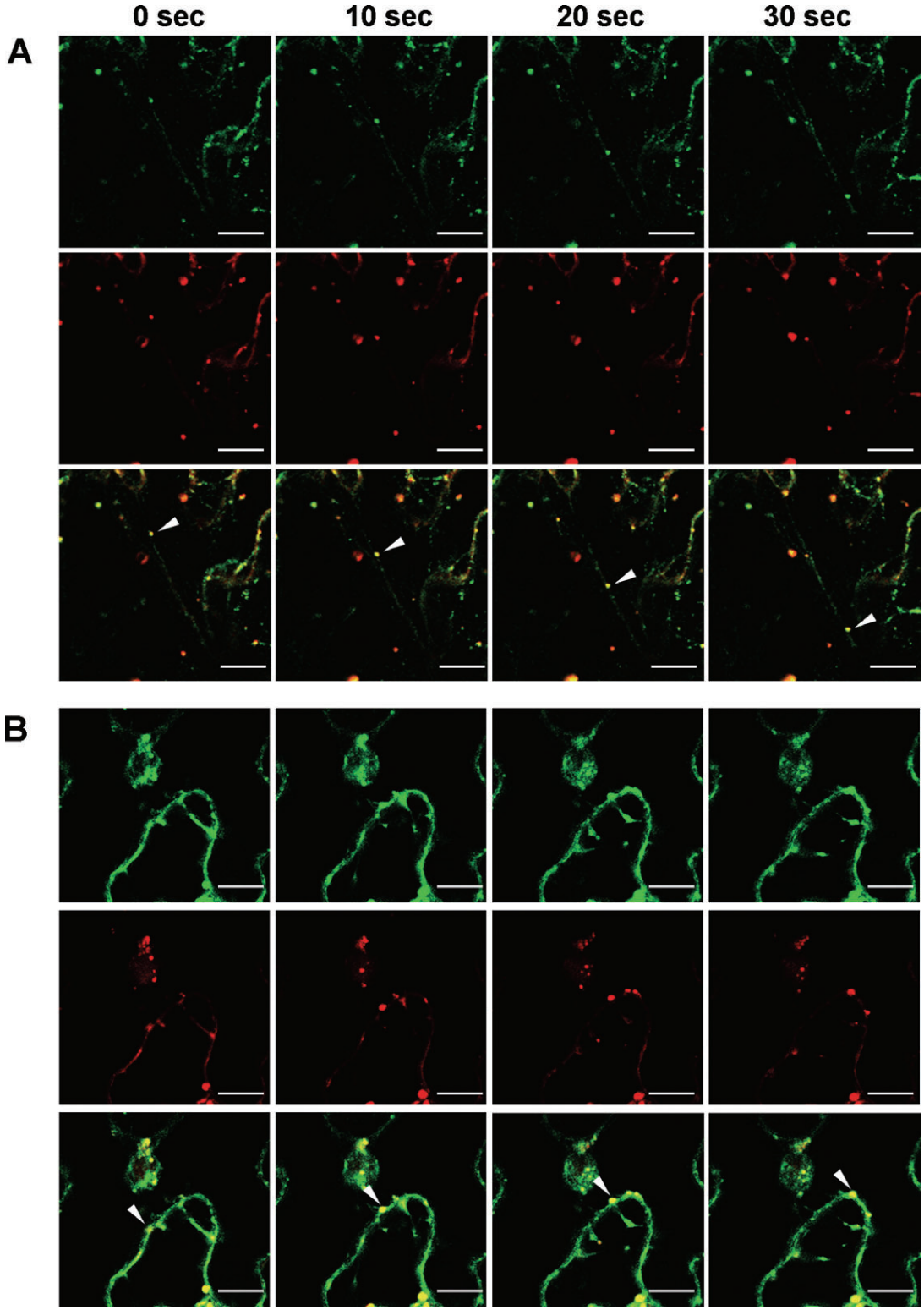

Fig. 2 Images recording the movement of p11-RFP in 16c epidermal cells. In each local field (A and B), four sequential pictures detecting green fluorescence and red fluorescence simultaneously were taken at 0, 10, 20 and 30 s. In (A) and (B), the top and middle lines of images show photographs taken under the green channel and red channel, respectively. The bottom line of images represents the merged photographs. White triangles point to the moving p11-RFP granules. Scale bar, 20 μm.

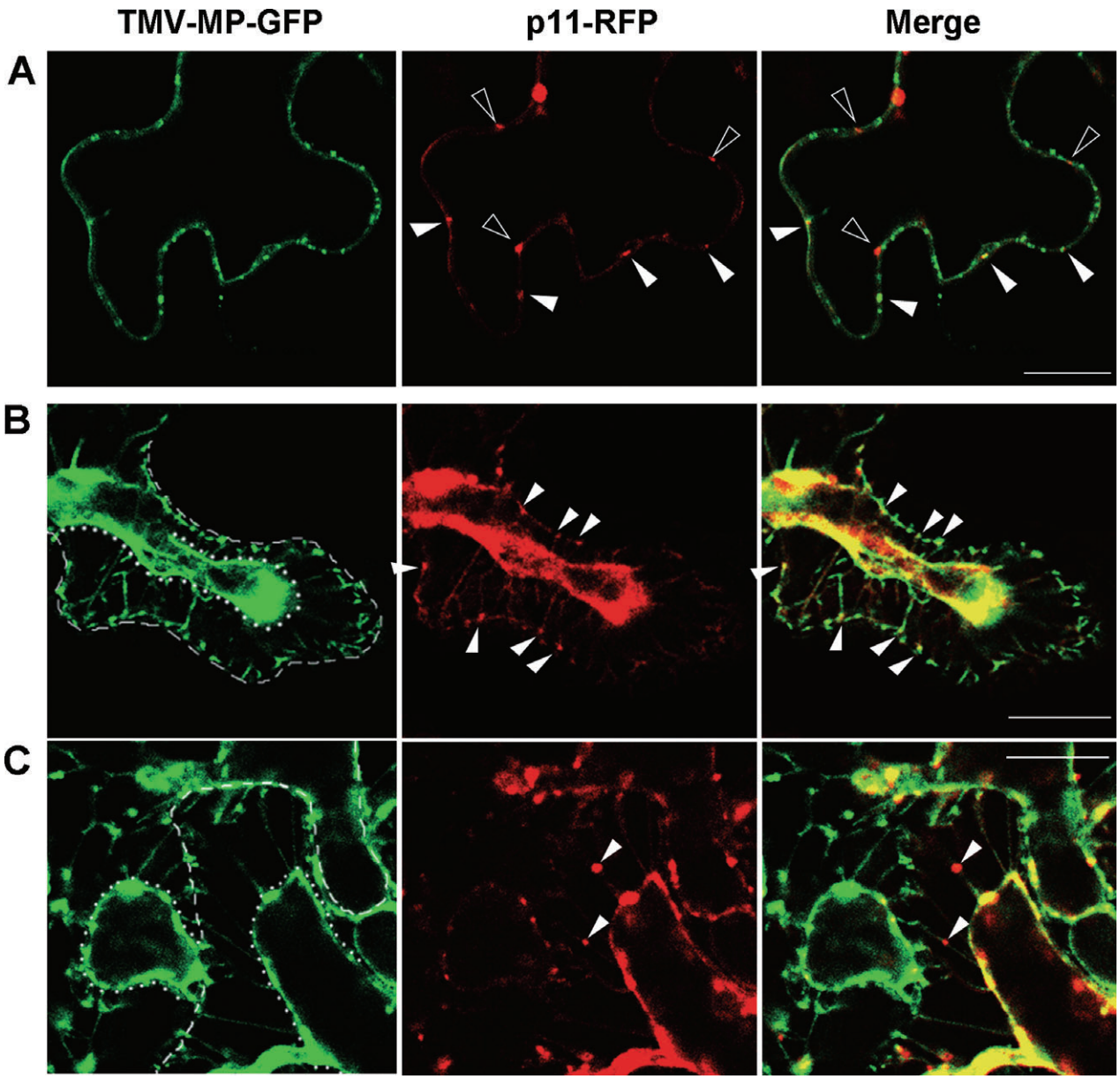

Fig. 3 Images showing the co-localization of p11-RFP (RFP, red fluorescent protein) and *Tobacco mosaic virus*-movement protein-green fluorescent protein (TMV-MP-GFP). (A) Localization of TMV-MP-GFP and p11-RFP on the cell periphery. White triangles and black triangles point to p11-RFP co-localized and not co-localized with TMV-MP-GFP, respectively. (B, C) Localization of TMV-MP-GFP and p11-RFP in plasmolysed cells. White triangles in (B) point to p11-RFP co-localized with TMV-MP-GFP. White triangles in (C) point to p11-RFP granules localized on strands between the cell wall and cytoplasm. The white broken and dotted lines outline the cell wall and cytoplasm, respectively. Scale bar, 20 μm.

co-expressed with p17-GFP in epidermal cells, the two proteins were shown to co-localize, demonstrating that p17 was localized to the nucleolus (Fig. 6D–F).

Effect of interaction on p11 and p17

When p17 was expressed alone, it was detected in the nucleolus (Fig. 6C), but, in the BiFC assay, where p17 and p11 were fused with the C-terminus and N-terminus of YFP, respectively, YFP fluorescence was visible in the cytoplasm and nucleus, but not in the nucleolus (Fig. 6A). This suggests that an interaction between p11 and p17 affects the location of p17. p11 was therefore co-expressed with p17-GFP in epidermal cells, with GUS and p17-GFP co-expressed to provide controls. In the control, fluorescence was visible in the nucleolus, similar to the pattern observed when p17-GFP was expressed alone (data not

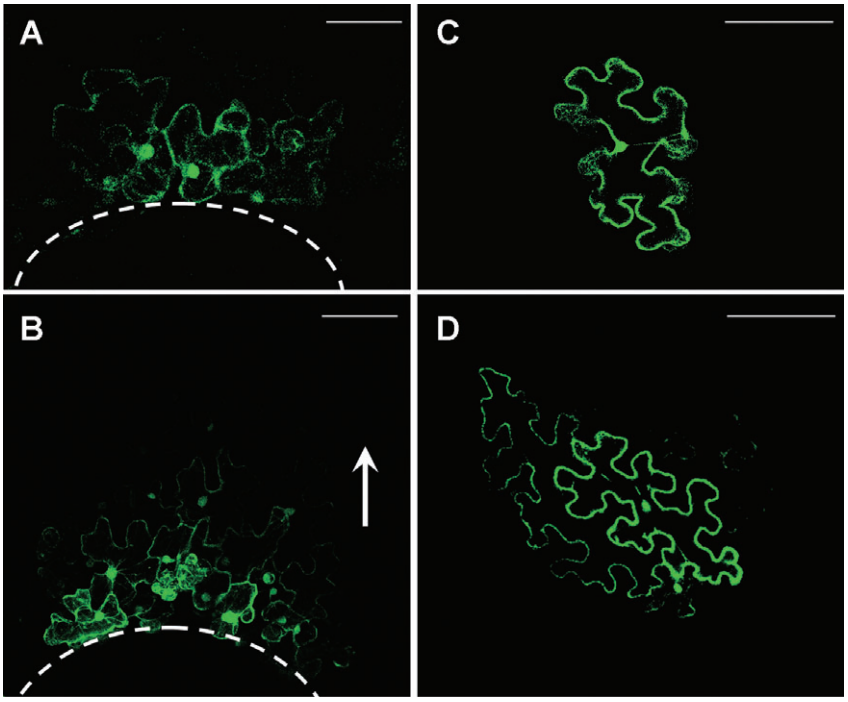

Fig. 4 Images showing the movement of p11-GFP (GFP, green fluorescent protein) from a single cell to adjacent cells after expression in *Nicotiana benthamiana* epidermal cells. (A) pΔGUS-GFP is restricted to the cells of the first layer. (B) Diffusion of p11-GFP into neighbouring cells after the infiltration of a small wound by *Agrobacterium*. (C, D) pΔGUS-GFP restricted to a single cell (C) and p11-GFP diffusing into neighbouring cells after bombardment (D). Scale bar, 100 μm.

Table 1 Rates of movement of fluorescence between bombarded cells and their neighbours.

Expressed protein	Fluorescence diffusion frequency*
pΔGUS-GFP	2/158 (~0)
p11-GFP	104/177 (~59)

*Data are presented as the number of bombarded sites containing two or more green fluorescent protein (GFP)-positive cells/total number of bombarded sites. Frequency (%) is shown in parentheses.

shown); however, in cells co-expressing p11 and p17-GFP, fluorescence occurred in the cell periphery, cytoplasm and nucleus, but not in the nucleolus, a pattern similar to that in the BiFC assay (Fig. 6G). However, when p11-RFP and p17-GFP were co-expressed, p11-RFP granules (which co-localized with p17-GFP in the cytoplasm) moved in the same manner as when p11-RFP was expressed alone (data not shown). These results show that GarVX p11 alters the location of garlic p17, but that movement of p11 is not affected by the interaction.

DISCUSSION

p11 is encoded by the third ORF of the GarVX genome and appears to correspond to TGB2 of carlaviruses, potexviruses and foveaviruses, although amino acid identities are not high (Table S1). This was confirmed by computer analysis showing that GarVX p11, like the TGB2 proteins from *Potato virus X* (PVX; *Potexvirus*), *Lily symptomless virus* (*Carlavirus*) and *Apple stem pitting virus* (*Foveavirus*), contains two TM segments (Fig. S1) (Rebelo *et al*., 2008; Samuels *et al*., 2007). In several experimental studies, GFP-fused TGB2 proteins have been shown to be associated with ER (Ju *et al*., 2005; Samuels *et al*., 2007). The experimental evidence of localization reported here further supports the view that p11 of GarVX is a membrane protein and localizes to ER.

Several studies have shown that all three TGB proteins are required for movement (Krishnamurthy *et al*., 2003; Leshchiner *et al*., 2006; Mitra *et al*., 2003; Samuels *et al*., 2007; Schepetilnikov *et al*., 2005; Tamai and Meshi, 2001; Verchot-Lubicz, 2005). Microinjection studies have indicated that TGB1 proteins of PVX and *White clover mosaic virus* (genus *Potexvirus*) chaperone viral RNA and move through PD (Lough *et al*., 1998, 2000). Biolistic studies have confirmed that the PVX TGB1-GFP fusion can facilitate its own cell-to-cell movement (Yang *et al*., 2000). Subcellular targeting analysis has revealed that TGB1 protein localizes in PD (Howard *et al*., 2004). These results suggest that the TGB1 protein has a direct role in viral cell-to-cell movement. TGB2 and TGB3 proteins are also important in intracellular trafficking of the virus (Krishnamurthy *et al*., 2003; Mitra *et al*., 2003; Tamai and Meshi, 2001). Mutational analysis has shown that TGB2 protein is necessary for PVX transport through PD, but is not sufficient on its own for viral cell-to-cell movement (Ju *et al*., 2007). The TGB3 protein has a TM segment at its N-terminus (Ju *et al*., 2008), and confocal and electron microscopy have shown that TGB3 is localized to ER (Bamunusinghe *et al*., 2009; Ju *et al*., 2008). A mutation disrupting the TM

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1   CGCTCCTGTCCCCATAAATACCATTAAAATCTCGAGAAAACAAACAAATTGAAATGGAGA
                                                          M  E

61  AGTTCAATGGAGGTTTGAAAGGCTACTGGGAACGCCGCGCCTACTCCCGCATCGACGGAG
    K  F  N  G  G  L  K  G  Y  W  E  R  R  A  Y  S  R  I  D  G

121 TTTCCGGCCGCCGCCGACCACGAGGCAGCAGAATCAAGCTCGGCGGTGGCACCGTCTCAC
    V  S  G  R  R  R  R  P  R  G  S  R  I  K  L  G  G  G  T  V  S

181 GCCGCCGCTGGCGAATCAGATTACCTAAAAAATTGAAGACAATACGGTTCCGGTTCAACC
    R  R  R  W  R  I  R  L  P  K  K  L  K  T  I  R  F  R  F  N

241 CGAAGCGGTTTTTCGCCAAGATCCGCGACGCCTATGTGCGGATGATGTTAGGGTTTGCTG
    P  K  R  F  F  A  K  I  R  D  A  Y  V  R  M  M  L  G  F  A

301 ACATGGGCGCAATTTCTGGCGGATTCGGGTACGCTGGGAAAGGGGTCGACGTGTTTGGGG
    D  M  G  A  I  S  G  G  F  G  Y  A  G  K  G  V  D  V  F  G

361 TCGAAAATAGAAAAATGAAGGAGTACGATGAAAAAATGCTGGTTCAGATTTACAGGAATC
    V  E  N  R  K  M  K  E  Y  D  E  K  M  L  V  Q  I  Y  R  N

421 TTTTGGTGCAGAGGCCGGATTTAGTCCCAACGGTCGATGATGGTGTGCCGGCGGTGGTGG
    L  L  V  Q  R  P  D  L  V  P  T  V  D  D  G  V  P  A  V  V

481 GTACCAGGGCGGCACCGATGACTGTTGTCTGAGATTTAAATGGCAGGTTTGTAATTTTAG
    G  T  R  A  A  P  M  T  V  V  *

541 AGGAAGTGAAGTGAAGTTTATGGATGGTGGTTGTATTATGTAAGTTCTTTAATCTATGCC

601 TAAGACAATAGTTCAGTTCGAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
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Fig. 5 The full cDNA sequence and amino acid sequence of garlic p17 (Accession Number: HM326891). The shadowed region represents the putative nuclear localization signal.

segment of the TGB3 protein eliminates ER association and inhibits virus cell-to-cell movement (Ju *et al*., 2008). With its similar protein structure and subcellular localization to other TGB2 proteins and its pattern of movement in the epidermal cells of *N. benthamiana*, p11 of GarVX appears to have the expected properties of a movement protein. How it interacts with ORF2 (the TGB1 homologue) is not known, and it is unclear whether a TGB3 homologue is expressed from the genome. A TGB3-like sequence can be identified in GarVX and other allexiviruses, but the usual AUG start codon is missing.

Several recent studies have reported the involvement of nucleolar proteins with plant viruses. Fibrillarin, a major nucleolar protein, interacts with the ORF3 protein of *Groundnut rosette virus* (GRV) and the nuclear inclusion protein a (NIa) of *Potato virus A* (Canetta *et al*., 2008; Kim *et al*., 2007; Rajamaki and Valkonen, 2009). Silencing of the Fib gene prevents long-distance movement of GRV, but does not affect viral replication or cell-to-cell movement (Kim *et al*., 2007). Nsr1p, another nucleolar protein involved in rRNA maturation and ribosome assembly, is capable of binding the 3′-UTR of tombusvirus RNA (Jiang *et al*., 2010). Overexpression of Nsr1p inhibits the accumulation of tombusvirus RNA by approximately 10-fold (Jiang *et al*., 2010). In our results, a nucleolar protein, p17 of garlic, was identified to interact with p11 of GarVX. BiFC assay confirmed their interaction in plant cells (Fig. 6A). Because the functions of all putative homologues of p17 are unknown and there is no viable virus-induced gene silencing (VIGS) system for garlic, it is unknown what role is played by p17 in plants, or whether p11 affects its function through their interaction. However, our results indicate, at least, that p11 alters the location of p17 (Fig. 6G), which makes it probable that there is a consequent effect on the normal function of p17.

EXPERIMENTAL PROCEDURES

Bacterial strains and plasmids

Construction of all plasmids followed standard cloning techniques (Sambrook *et al*., 1989) and *Escherichia coli* strain TG1 was used for transformation. All constructed plasmids were from pCV1300 backbones and are shown in Fig. 7. pCV1300 was derived from pCAMBIA1300 (CAMBIA, Canberra, Australia) after insertion of the 35S promoter-GUS-Nos terminator frame from pBI121 into pCAMBIA1300 using *Hind*III and *EcoR*I. p11, which was introduced into pGEM-T (Promega, Madison, WI, USA) with forward primer 5′-*GGATCC*ATGAGCTTCACTCCCCC GC-3′ (*BamH*I site in italic) and reverse primer 5′-*GAGC TC*TTAGTGGGGTAGAGTAGAT-3′ (*Sac*I site in italic), was digested with *BamH*I and *Sac*I, and ligated into pCV digested with the same enzymes to generate pCV:p11.

Before construction of the plasmid for targeting and BiFC assay, pCV:GFP, pCV:RFP, pCV:nYFP-C and pCV:cYFP-C backbones were constructed. GFP, amplified from Fgc-eGFP with the forward primer 5′-*TCTAGA*CCCCTG*GGATCC*TGCCTG*CCCGGG* GCCTGG*GGTACC*CTGTGC*GTCGAC*ATGGTGAGCAAGGGCGAGG AGC-3′ (*Xba*I, *BamH*I, *Sma*I, *Kpn*I and *Sal*I sites in italic in that order) and reverse primer 5′-*GAGCTC*TCACACCTTGTAACAG CTCTGTC-3′ (*Sac*I site in italic) was cloned into the pGEM-T

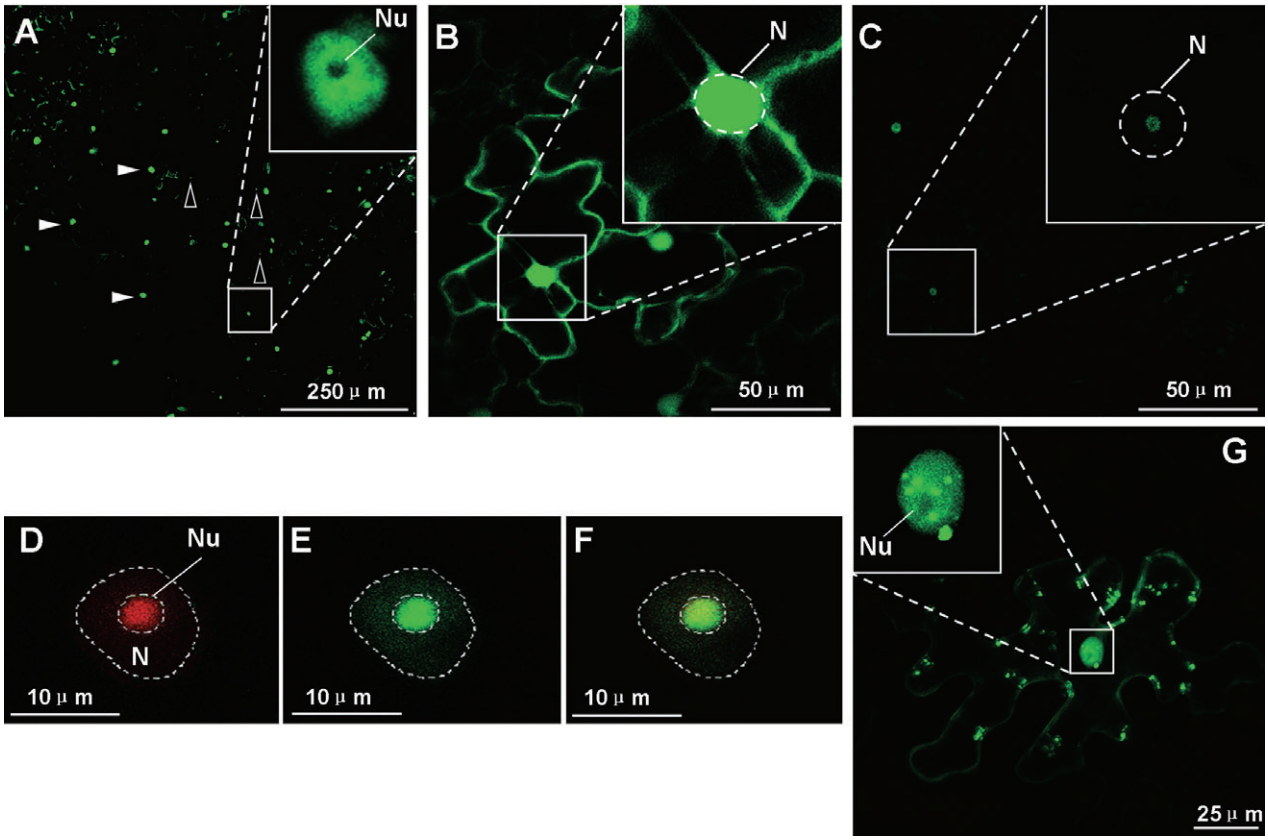

Fig. 6 Images from bimolecular fluorescence complementation (BiFC) assay showing p11 and p17, and the localization of p17. (A) Fluorescence in the BiFC assay. The fluorescence-filled nucleus has been enlarged to shown that fluorescence was not in the nucleolus. (B, C) Nucleolar localization of p17. Compared with control cells expressing pΔGUS-GFP (B), where fluorescence was distributed in the cytoplasm and nucleus, in cells expressing p17-GFP (C), fluorescence was restricted to the nucleolus. Nuclei are enlarged. (D–F) Confirmation of the localization of p17 in the nucleolus. (D) Nucleolar localization of Fib-2 fused to red fluorescent protein (RFP). (E) Localization of p17 fused to green fluorescent protein (GFP). (F) Merged images of (D) and (E). The nucleus (N) and nucleolus (Nu) are outlined with a white broken line. (G) Change in p17-GFP localization from nucleolus to nucleus and cytoplasm when co-expressed with p11. The nucleus is enlarged.

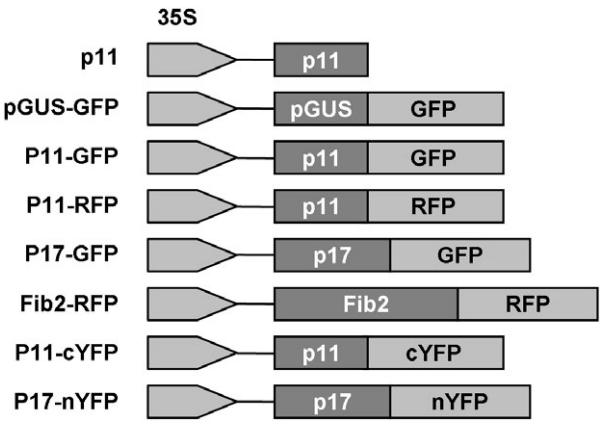

Fig. 7 Diagram showing the plasmids used in this work. They were constructed in the pCAMBIA1300 binary vector driven by the CaMV 35S promoter.

vector, creating pGEM:GFP. pCV:GFP was constructed by digesting GFP from pGEM:GFP with *Xba*I and *Sac*I, and ligating it with pCV1300 digested with the same enzymes. pCV:RFP was constructed similarly, but with forward primer 5′-*TCTAGA*CCCC TG*GGATCC*TGCCTG*CCCGGG*GCCTGG*GGTACC*CTGTGC*GTCGAC* ATGGTGCGCTCCTCCAAGAACGTC-3′ (*Xba*I, *Bam*HI, *Sma*I, *Kpn*I and *Sal*I sites in italic in that order) and reverse primer 5′-*GAGCT*CTTAAGATCTGAGCAGGAACAGG-3′ (*Sac*I site in italic). RFP was from pDsred1-C1 (Clontech, Mountain View, CA, USA). nYFP amplified from SAT1-nEYFP-C1 with the forward primer 5′-*TCTAGA*CGAGAGTTCTCAACACAACATATACAAAAC-3′ (*Xba*I site in italic) and reverse primer 5′-*GAGCTC* GCCTGG*GGATCC*GCATCT*CCCGGG*GCCTGG*GGTACC*CTGTGC*GT CGAC*TCCGGAGTCCTCGATGTTGTGGC-3′ (*Sac*I, *Bam*HI, *Sma*I, *Kpn*I and *Sal*I sites in italic in that order) was cloned into the pGEM-T vector, creating pGEM:nYFP. pCV:nYFP was constructed by digesting nYFP from pGEM:nYFP with *Xba*I and *Sac*I, and

ligating it with pCV1300 digested with the same enzymes. pCV:cYFP was constructed similarly using cYFP from SAT1-cEYFP-C1 and with forward primer 5′-*TCTAGA*CGAGAGTT CTCAACACAACATATACAAAAC-3′ (*Xba*I site in italic) and reverse primer 5′-*GAGCTC*GCCTGG*GGATCC*GCATCT*CCCGGG*GCCTG G*GGTACC*CTGTGC*GTCGAC*TCCGGACTTGTACAGCTCGTCCAT-3′ (*Sac*I, *Bam*HI, *Sma*I, K*pn*I and *Sal*I sites in italic in that order). Both SAT1-nEYFP-C1 and SAT1-cEYFP-C1 were kindly provided by Dr Gelvin (Purdue University, West Lafayette, IN, USA).

For construction of pCV:p11-GFP and pCV:p11-RFP, p11 was introduced into pGEM-T with forward primer 5′-*TCT AGA*ATGAGCTTCACTCCCCCG-3′ (*Xba*I site in italic) and reverse primer 5′-*GTCGAC*GTGGGGTAGAGTAGATTC-3′ (*Sal*I site in italic), creating pGEM:p11-b. p11 was then digested from pGEM:p11-b with *Xba*I and *Sal*I, and ligated into pCV:GFP and pCV:RFP both digested with the same enzymes. The partial GUS encoding sequence that contained equal nucleotide numbers to that of p11 was amplified from pBI121 with the forward primer 5′-*GGATCC*CGTCCTGTAGAAACCCCAACCCGT-3′ (*Bam*HI site in italic) and reverse primer 5′-*GTCGAC*CGGTATAAAGACTTC GCGCTG-3′ (*Sal*I site in italic), and then cloned into pCV:GFP to create pCV:pΔGUS-GFP in the same manner as used to generate pCV:p11-GFP. Similarly, pCV:p17-GFP was constructed with the forward primer 5′-*GGATCC*ATGGAGAAGTTCAATGGAGGT-3′ (*Bam*HI site in italic) and reverse primer 5′-*GTCGAC* GACAACAGTCATCGGTGCCGCCC-3′ (*Sal*I site in italic).

For BiFC assay of p11 and p17, pCV:p11-cYFP and pCV:p17-nYFP were constructed. p11 was introduced into pGEM-T with forward primer 5′-*GTCGAC*ATGAGCTTCACTCCCCCG-3′ (*Sal*I site in italic) and reverse primer 5′-*GGATCC*GTGGGGTAGA GTAGATTC-3′ (*Bam*HI site in italic), creating pGEM:p11-c. p11 was digested from pGEM:p11-c with *Sal*I and *Bam*HI, and ligated into pCV:cYFP digested with the same enzymes, generating pCV:p11-cYFP. p17 was introduced into pGEM-T with forward primer 5′-*GTCGAC*ATGAGCTTCACTCCCCCG-3′ (*Sal*I site in italic) and reverse primer 5′-*GGATCC*GTGGGGTAGAGTAGATTC-3′ (*Bam*HI site in italic), creating pGEM:p17-c. p17 was digested from pGEM:p17-c with *Sal*I and *Bam*HI, and ligated into pCV:nYFP digested with the same enzymes, generating pCV:p17-nYFP. In the same way, pCV:pΔGUS-nYFP and pCV:pΔGUS-cYFP were produced for control.

The Fib gene (Accession Number: AM269909) was amplified from *N. benthamiana* cDNA with the forward primer 5′-*TCTAGA*ATGGTTGCACCAACTAGAGGTCG-3′ (*Xba*I site in italic) and reverse primer 5′-*GGATCC*GGCAGCAGCCTTCT GCTTC-3′ (*Bam*HI site in italic). The PCR product was purified and cloned into pGEM-T, creating pGEM-Fib. Fib digested from pGEM-Fib with *Xba*I and *Bam*HI was ligated with pCV:RFP digested with the same enzymes, creating pCV:Fib-RFP.

To construct the plasmids used in the yeast two-hybrid system, p11 amplified from pGEM:p11-b with the forward primer 5′-*CATATG*ATGAGCTTCACTCCCCCG-3′ (*Nde*I site in italic) and reverse primer 5′-*GGATCC*TTAGTGGGGTAGAGTAGAT-3′ (*Bam*HI site in italic) was introduced into pGBKT7, producing pGBKT7:p11. The construction of a garlic cDNA library in the pGADT7 vector followed the method described previously (Shi *et al*., 2007).

Plant material, *Agrobacterium* infiltrations and bombardment

Nicotiana benthamiana and *N. benthamiana* line 16c were used to explore the subcellular targeting of proteins. These were grown as described by Voinnet *et al*. (1998). *Agrobacterium* strain C58C1 was used for *Agrobacterium* infiltration. For co-infiltrations, equal volumes of individual *Agrobacterium* cultures [optical density at 600 nm (OD_{600}) = 1.0] were mixed before co-infiltration. Bombardment was carried out with a Bio-Rad (Hercules, CA, USA) Heliox Gene Gun (100–600 psi) according to the manufacturer's instructions. Plasmolysis experiments were carried out as described by Tilsner *et al*. (2010).

Yeast two-hybrid assay

Matchmaker Yeast Two-Hybrid System 3 (Clontech) was used for yeast two-hybrid assays to screen the garlic protein interacting with p11. The experiments were carried out according to the instructions provided and as described in our previous work (Shi *et al*., 2007). Briefly, yeasts transformed with pAD and pBD plasmids were grown on low-stringency medium (SD/–Leu/–Trp). Positive candidates were transferred to high-stringency medium (SD/–Leu/–Trp/–His/–Ade) containing X-α-Gal. The positive gene was then introduced into the pGADT7 vector for repeat of the yeast two-hybrid assay.

Microscopy

A Leica TCS SP5 (Leica Microsystems, Bannockburn, IL, USA) confocal laser scanning microscope system was used to examine the fluorescence of GFP, RFP and YFP. All images were processed using Adobe Photoshop version 7.0 software (Adobe Systems Inc., San Jose, CA, USA).

Sequence analysis

Alignment of the sequences was performed by CLUSTAL 2.0. TM helices of p11 and TGB2 proteins were predicted with TMHMM 2.0 (http://www.cbs.dtu.dk/services/TMHMM/). The potential NLSs of proteins were predicted with Motif Scan (http://myhits.isb-sib.ch/cgi-bin/motif_scan) (Hulo *et al*., 2008).

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Fig. S1 Transmembrane (TM) segment analysis of triple gene block 2 (TGB2) proteins from *Garlic virus X* (GarVX), *Potato virus X* (PVX) (*Potexvirus*), *Lily symptomless virus* (LSV) (*Carlavirus*) and *Apple stem pitting virus* (ASPV) (*Foveavirus*).

Fig. S2 Amino acid alignment of garlic p17 with the putative homologues from other species [*Arabidopsis thaliana* (NP_191824), *Oryza sativa* (NP_001042850), *Picea sitchensis* (ABK21989), *Populus trichocarpa* (XP_002302741), *Ricinus communis* (XP_002515331), *Sorghum bicolor* (XP_002457739), *Vitis vinifera* (XP_002274279) and *Zea mays* (NP_001159124)]. The conserved amino acids are underlined.

Table S1 Percentage amino acid identities between p11 of *Garlic virus X* (GarVX) and the triple gene block 2 (TGB2) proteins of carlaviruses, potexviruses and foveaviruses.

Video S1 Movement of p11-RFP under the red channel in cells of *N. benthamiana* 16c.

Video S2 Movement of p11-RFP under the green channel in cells of *N. benthamiana* 16c.

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