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Short title: PMTV co-opts HIPP26 for systemic infection

Corresponding author: Professor Lesley Torrance, University of St Andrews and The James Hutton Institute, Scotland, UK.

Title: Potato mop-top virus co-opts the stress sensor HIPP26 for long-distance movement

Graham H. Cowan[1], Alison G. Roberts[1], Susan Jones[1], Pankaj Kumar[2], Pruthvi B Kalyandurg [3], Jose F Gil[3], Eugene I Savenkov[3], Piers A. Hemsley[1,5] and Lesley Torrance[1, 2*]

[1] The James Hutton Institute, Invergowrie, Scotland, UK

[2]School of Biology, The University of St Andrews, St Andrews, Fife, UK

[3]Department of Plant Biology, Uppsala BioCenter, Linnean Centre of Plant Biology, Swedish University of Agricultural Sciences, Uppsala, Sweden

[5]Division of Plant Sciences, University of Dundee at the James Hutton Institute, Invergowrie, Scotland, UK

Summary sentence: After a virus movement protein interacts with the HIPP26 stress sensor, the complex re-localises to the nucleus, activating the drought stress response and thereby facilitating virus long distance movement.

Footnotes

Author contributions: LT conceived the research plans; LT, PH, and EIS designed the research; GC, SJ, PK, PBK, JFG, AR, and PH performed the research; GC, LT, AR, PH, SJ, and EIS analyzed the data; and LT, AR, PH, and EIS wrote the paper with contributions from all authors.

Funding: The work of LT, GC, SJ and AR is funded by the Scottish Government's Rural and Environmental Science and Analytical Services (RESAS) Division, PH by the BBSRC (grant BB/M024911/1) and The Royal Society and EIS by the Swedish Research Council Formas and the Carl Tryggers Foundation.

***Corresponding author email**: Lesley.Torrance@hutton.ac.uk; lt27@st-andrews.ac.uk

The authors responsible for distribution of materials integral to the findings presented in this article are: Lesley Torrance (Lesley.Torrance@hutton.ac.uk) and Eugene Savenkov (eugene.savenkov@slu.se)

Abstract

Virus movement proteins facilitate virus entry into the vascular system to initiate systemic infection. The potato mop-top virus (PMTV) movement protein, TGB1, is involved in long-distance movement of both viral ribonucleoprotein complexes and virions. Here, our analysis of TGB1 interactions with host *Nicotiana benthamiana* proteins revealed an interaction with a member of the *h*eavy metal-associated *i*soprenylated *p*lant *p*rotein family, HIPP26, which acts as a plasma membrane-to-nucleus signal during abiotic stress. We found that knockdown of *NbHIPP26* expression inhibited virus long-distance movement but did not affect cell-to-cell movement. Drought and PMTV infection upregulated *NbHIPP26* gene expression, and PMTV infection protected plants from drought. In addition, *NbHIPP26* promoter-reporter fusions revealed vascular tissue-specific expression. Mutational and biochemical analysis indicated that *Nb*HIPP26 sub-cellular localisation at the plasma membrane and plasmodesmata was mediated by lipidation (S-acylation and prenylation), as non-lipidated *Nb*HIPP26 was predominantly in the nucleus. Notably, co-expression of *Nb*HIPP26 with TGB1 resulted in a similar nuclear accumulation of *Nb*HIPP26. TGB1 interacted with the C-terminal CVVM (prenyl) domain of *Nb*HIPP26, and bimolecular fluorescence complementation revealed that the TGB1-HIPP26 complex localized to microtubules and accumulated in the nucleolus, with little signal at the plasma membrane or plasmodesmata. These data support a mechanism where interaction with TGB1 negates or reverses *Nb*HIPP26 lipidation, thus releasing membrane-associated *Nb*HIPP26 and re-directing it via microtubules to the nucleus, thereby activating the drought stress response and facilitating virus long-distance movement.

INTRODUCTION

The systemic infection of plants by viruses results in major economic losses in yield and/or quality of harvested tissues (Pennazio et al., 1996; Waterworth and Hadidi, 1998). To achieve a systemic infection, viruses must move from the initial point of entry to neighbouring cells and then long distance in the vasculature to other leaves and organs (reviewed in Tilsner et al., 2014). Although there has been much research on the mechanisms of virus cell-to-cell

movement, there is much less knowledge of the molecular details of how viruses access the vasculature and the phloem mass transport pathway for long distance movement. A better understanding of this process is needed to develop new interventions for disease control.

The plant vasculature is a major conduit not only for nutrient and water transfer but also for the perception and response to abiotic and biotic stresses (Kehr and Buhtz, 2008). Plant proteins destined for phloem transport are made in, or transferred to, companion cells prior to transfer into sieve elements through specialised plasmodesmata (PD) called pore plasmodesmal units (PPU) (reviewed in Lough and Lucas, 2006). The phloem contains many different macromolecules including protein chaperones, calcium sensors, RNA binding proteins (Giavalisco et al., 2006) and different species of RNAs (including mRNAs, miRNAs and siRNAs) (Buhtz *et al.* 2008, 2010*;* reviewed by Turgeon and Wolf, 2009). These phloem-mobile signalling molecules enable plants to coordinate their growth and development and respond to stress (Kehr and Buhtz, 2008). A recent report on systemic movement of *Tobacco mosaic virus* (TMV) suggests that interaction with phloem-specific transcriptional regulators plays a major role in virus phloem loading in older tissues suggesting that TMV reprogramming of gene expression enhances systemic spread (Collum et al., 2016).

In this paper we studied the long-distance movement of *Potato mop-top virus* (PMTV), the type member of the genus *Pomovirus*. PMTV is one of only a few plant viruses where, in addition to movement as encapsidated virions, the genome components can also move as ribonucleoprotein (vRNP) complexes, and the largest movement protein, TGB1, plays a key role in the long-distance movement of both (Torrance et al., 2011). Previous work has shown that the N-terminal domain of TGB1 contains two nucleolar localisation signals, and TGB1 associates with importin-α, which is required for nuclear/nucleolar accumulation and virus long distance movement (Lukhovitskaya et al., 2015). The nucleolus is a distinct region in the nucleus that has recently been shown to play a role in long distance movement of some plant viruses (reviewed in Taliansky et al., 2010). For example, *Groundnut rosette virus* (GRV) does not encode a coat protein but encodes the protein ORF3 that interacts with a nucleolar component, fibrillarin, to form movement competent vRNP for long distance transport (Kim et al., 2007).

Here we describe the association of a virus movement protein, PMTV TGB1, with a vascular-expressed plant stress sensor HIPP26. HIPP26 is one member of a family of proteins that contain heavy metal binding and C-terminal isoprenylation motifs (heavy metal-associated

isoprenylated plant protein; HIPP). HIPPs are unique to vascular plants and form a large and diverse family in Arabidopsis (*Arabidopsis thaliana*; Dykema et al., 1999; Barth et al., 2009; Tehseen et al., 2010; de Abreu-Neto et al., 2013). They have been shown to act in heavy metal homeostasis and in regulating the transcriptional response to abiotic stress (de Abreu-Neto et al., 2013; Barth et al., 2009; Gao et al., 2009) and pathogens (de Abreu-Neto et al., 2013). Our results show that PMTV TGB1 interacts with HIPP26 and the complex accumulates in the nucleus which leads to the activation of the drought stress response pathway in vascular tissues, which in turn facilitates the access of PMTV to the transport phloem for long distance movement.

RESULTS

PMTV TGB1 interacts with tobacco (*Nicotiana benthamiana*) HIPP26

To investigate interactions of TGB1 with host factors, an *N. benthamiana* cDNA library (Hybrigenics Services SAS) was screened by the yeast two-hybrid (Y2H) system and 50 clones were identified as interacting with TGB1. The results were returned with each interacting clone provided with a predicted biological score (Formstecher et al., 2005) ranked in categories A to F, with A as the highest level of confidence in the specificity of the interaction. Twenty-nine clones were ranked A, and by using BLAST (Altschul et al., 1990) they were all identified as *N. benthamiana* homologues of Arabidopsis HIPP26. A representative clone, A44, which contained the near complete sequence (missing 33 5' proximal base pairs coding for 11 N-terminal amino acid residues) of the *N. benthamiana* homologue of Arabidopsis HIPP26 was obtained from Hybrigenics. In addition, the complete open reading frame (ORF) was cloned from *N. benthamiana* plants and sequenced.

The cDNA sequence of *Nb*HIPP26 ORF was used to search the predicted proteins from the draft *N. benthamiana* genome (assembly v1.01) using Blast through the Sol Genomics Network web server (http://solgenomics.net) (Fernandez-Pozo et al., 2014) This revealed significant matches on two different scaffolds (Niben101Scf07109g05004.1 and Niben101Scf02621g04026.1) that have not yet been assigned to any chromosomes. The two proteins only differed in one amino acid residue at position 16 near the N-terminus, which was either a serine or glycine. Further genome assembly and annotation is required to confirm whether the proteins are coded by alleles or distinct genes. In this work we have investigated

the protein containing S^{16} since serine was the most common residue at that position in the interacting clones.

HIPPs have been variously clustered into five (de Abreu-Neto et al., 2013), six (Barth et al., 2009) and seven major groups (Tehseen et al., 2010). The phylogeny created from the alignment of the interacting clone with full length Arabidopsis HIPPs reveals five clusters similar to those identified by de Abreu-Neto and co-workers (de Abreu-Neto et al., 2013) (Fig. 1A; Supplemental Fig. 1). In this phylogeny, clone A44 clusters with *At*HIPP26 in HIPP group II which features proteins with a single heavy metal associated (HMA) domain. Sequence alignments of *Nb*HIPP26 with homologues from Arabidopsis, tomato (*Solanum lycopersicum*) and potato (*Solanum tuberosum*) revealed a high level of conservation; with 84.3% sequence identity between *N. benthamiana* and Arabidopsis, 95.4% identity between *N. benthamiana* and potato, and 94.8% between *N. benthamiana* and tomato sequences (Fig. 1B and C).

Analysis revealed that the $C^{152}VVM^{155}$ sequence at the C-terminus of *Nb*HIPP26 represents a CaaX amino acid motif (a = I, V, L, A, S, T; X = I, L, M, Q, S, A) characteristic of proteins that are post-translationally lipid modified by the addition of a prenyl group to enable membrane association (reviewed in Crowell, 2000). *At*HIPP7 was previously shown to be prenylated *in vitro* through a similar CTVM motif (Dykema et al 1999) while mammalian N-Ras is known to be prenylated at an identical CVVM motif *in vivo* and the CVVM motif alone fused to GFP is sufficient to target GFP to membranes (Choy et al., 1999). Plant proteins in the HIPP family all contain a CaaX motif and are all thought to be prenylated (de Abreu-Neto et al., 2013). Furthermore, a strong prenylation prediction was obtained when the *Nb*HIPP26 sequence was analysed using Prenylation Prediction Suite software (Score = 2.514, P = 0.000554; Maurer-Stroh and Eisenhaber, 2005). These data combined indicate that *Nb*HIPP26 is likely prenylated and we therefore term the HIPP26 CVVM motif a prenylation (or prenyl) motif.

The full length *Nb*HIPP26 ORF was cloned and tested again in Y2H, confirming the interaction with PMTV TGB1 (Fig. 2A). In addition, the movement proteins of four other viruses were tested to investigate whether the interaction with PMTV TGB1 was specific or whether other virus movement proteins might also interact with *Nb*HIPP26. These proteins were chosen to represent common types of virus movement proteins (Tilsner et al., 2014; Verchot-Lubicz et al., 2010), including a) beet virus Q (BVQ) TGB1 representing another pomovirus, b) barley stripe mosaic virus (BSMV) TGB1; a 'hordei-type' TGB1 protein with a helicase domain and

large N-terminal (RNA binding) extension which does not share similarity with the N-terminal domain of PMTV TGB1, c) potato virus X (PVX) TGB1 representing a 'potex-type' TGB which contains a helicase domain but not an N-terminal extension, and d) tobacco mosaic virus (TMV) 30K movement protein representing the extensively studied 30K family. These were cloned into the Y2H system, but while PMTV TGB1 interacted with *Nb*HIPP26, no interactions were obtained with the other virus movement proteins (Fig. 2A). The BSMV, BVQ, TMV, and PVX movement proteins were detected by immunoblot in the yeast clones confirming that the proteins were expressed in yeast.

TGB1 interacts with the C-terminus of *Nb*HIPP26

To identify key residues involved in the interactions, mutations were introduced into PMTV *TGB1* and the predicted functional domains of *NbHIPP26*. The *TGB1* mutants were previously shown to produce N-terminally truncated TGB1 proteins lacking amino acid residues 1-84 and 1-149 (TGB1 Δ84 and Δ149 respectively; Wright et al., 2010; Lukhovitskaya et al., 2015). In *Nb*HIPP26, the prenyl motif, $C^{152}VVM^{155}$, was altered by replacing C^{152} with glycine, which would interrupt any possible prenylation and was referred to as $Prenyl^{M}$. The HMA domain ($M^{36}DCEGC^{41}$) was altered by replacing the $C^{38,41}$ residues with glycine residues to give HMA^{M} (Fig. 2B).

In Y2H assays, TGB1 Δ84 interacted with *Nb*HIPP26 but the truncated protein TGB1 Δ149 did not (Fig. 2C), suggesting that some or all of the interacting surface is within the TGB1 region spanning amino acid residues 85 to 149. This idea was further supported by the lack of interaction between *Nb*HIPP26 and BVQ TGB1, the movement protein of a related virus which showed very little similarity in the $TGB1^{85\text{-}149}$ fragment (Fig. 2D). In general, the C-terminal part of pomoviral TGB1 proteins such as those from PMTV and BVQ was well conserved and comprised the helicase domain, whereas the N-termini of the proteins showed very little similarity. The $Prenyl^{M}$ altered protein did not interact with TGB1 whereas HMA^{M} retained the interaction (Fig. 2C), indicating that the site containing the CVVM motif was important for binding to TGB1.

GFP-*Nb*HIPP26 localises in several sub-cellular domains

To investigate the localisation of *Nb*HIPP26, the protein was expressed as a translational fusion to GFP in *N. benthamiana* epidermal cells. Green fluorescence was observed in the plasma membrane, small motile vesicles (approx. 2 µm diameter; Supplemental Movie S1), the

nucleoplasm, and the nucleoli (Fig. 3A). Co-expression with mRFP-fibrillarin (a marker for the nucleolus; Goodin et al., 2007) showed precise co-localisation (Fig. 3B). Expression of GFP-*Nb*HIPP26 in *N. benthamiana* plants transformed with the mOrange-LTi6b plasma membrane marker (Wright et al., 2017) showed strong co-localisation of GFP and mOrange signal as well as punctate spots of GFP-*Nb*HIPP26 around the cell periphery which resembled PD (Fig. 3C). To determine whether these spots were PD, GFP-*Nb*HIPP26 was co-expressed with the PD-localised TMV 30K movement protein fused to mRFP. Both fluorescent proteins co-localised in some punctate spots at the periphery (Fig. 3D) showing that *Nb*HIPP26 localises to a population of PDs. Thus, the results show that *Nb*HIPP26 has several subcellular localisations including PD, plasma membrane, motile vesicles, nucleoplasm and nucleolus.

The TGB1-*Nb*HIPP26 complex accumulates in the nucleolus

To investigate the TGB1-*Nb*HIPP26 interaction in plant cells, BiFC experiments were conducted using split YFP constructs (Martin et al., 2009). *NbHIPP26* and *TGB1* genes were cloned to allow expression of *Nb*HIPP26 fused to the N-terminal domain of YFP (Y-*Nb*HIPP26) and TGB1 fused to the C-terminal domain (TGB1-FP). When these constructs were co-expressed in *N. benthamiana* epidermal cells, yellow fluorescence was seen labelling microtubules and nucleoplasm with particularly strong yellow fluorescence in the nucleolus (Fig. 3E and F). GFP-HIPP26 never localised to microtubules when expressed alone. The TGB1 N-terminally-truncated derivatives Δ84 and Δ149 were also tested in the BiFC system, and the results showed that yellow fluorescence was reconstituted in the nucleus and nucleolus but not on microtubules with the combination Δ84-FP and Y-*Nb*HIPP26 (Fig. 3G) but no complementation was observed in tests with Δ149-FP and Y-*Nb*HIPP26 (data not shown; no fluorescence was visible). No complementation (no fluorescence) was observed in control experiments with Y-*Nb*HIPP26 or TGB1-FP and the corresponding empty vectors encoding Y- or FP fragments. Both HIPP26 and TGB1 localise in the nucleus (Barth et al., 2009; Lukhovitskaya et al., 2015 respectively); however, the BiFC data show that the TGB1-*Nb*HIPP26 complex also localises to microtubules. This result taken together with the absence of visible fluorescence at the plasma membrane and PD indicated that the interaction with TGB1 caused a microtubule-guided re-localisation of HIPP26 from the plasma membrane to the nucleus and nucleolus. This result recapitulates the TGB1 distribution pattern seen previously with YFP-TGB1 behind the leading edge of the virus infection (Wright et al., 2010).

To support the BiFC data, GFP-HIPP26 was also co-expressed in epidermal cells with RFP-TGB1. In these experiments green and red fluorescence co-localized precisely in the nucleoplasm and nucleolus and on microtubules (Fig. 3H, I and J). We also expressed GFP-HIPP26 in PMTV-infected cells, and in these cells GFP fluorescence was localised as described before when expressed in non-infected cells but it also accumulated on microtubules (Fig. 3K). Note that GFP-HIPP26 was never seen on microtubules when expressed alone. Thus we believe these data support the BiFC results and provide confirmatory evidence of the interaction between HIPP26 and TGB1 in plants.

*Nb*HIPP26 is an S-acylated protein

Proteomic studies have revealed that many Arabidopsis proteins involved in abiotic or biotic stresses can be reversibly lipid modified by S-acylation (Hemsley et al., 2013). S-acylation involves the post-translational addition of fatty acids, predominantly stearic or palmitic acid, to cysteine residues within proteins through a reversible thioester linkage (Sorek et al., 2007). Various Arabidopsis HIPP family members were proposed as S-acylated based on proteomics data (Hemsley et al., 2013). We therefore tested whether GFP-*Nb*HIPP26 expressed in *N. benthamiana* was S-acylated using a modified Acyl-RAC assay (Hemsley et al., 2008, Forrester et al., 2011). The results revealed that *Nb*HIPP26 was indeed S-acylated (Figure 4). Substitution of candidate S-acylated cysteine resides by glycine, alanine, or serine is the most common way to identify S-acylation sites. *Nb*HIPP26 contains four cysteine residues; one as the prenyl acceptor in the CVVM motif, two in the HMA domain presumed to act as metal ion coordinators, and one of unknown function at the N-terminus (C^{13}). On the basis that C^{13} is the only cysteine residue with no proposed function, we replaced it with glycine ($C^{13}G$). Subsequent quantification of S-acylation assays comparing the $C^{13}G$ and $Prenyl^{M}$ *Nb*HIPP26 to wild type (Figs. 2B and 4A) indicated that the $C^{13}G$ altered protein showed almost total loss of S-acylation signal and C^{13} was therefore the likely site of S-acylation. The $C^{13}G$ altered protein is henceforth referred to as $S\text{-}Acyl^{M}$. Prior membrane association is thought to be required for maximal S-acylation efficiency by the integral membrane S-acylating enzymes. The small but significant (n = 2, P = 0.103) decrease in the S-acylation state of *Nb*HIPP26 altered protein $Prenyl^{M}$ compared with wild type (Figure 4B) indicated that non-prenylated *Nb*HIPP26 was still a substrate for S-acylation. This suggests that HIPP26 can interact with membranes through means other than prenylation and S-acylation.

*Nb*HIPP26 proteins altered in the lipidation domains show depletion from membranes and nuclear enrichment

To determine whether lipidation is needed for *Nb*HIPP26 binding to the plasma membrane and PD, the subcellular localisations of GFP-tagged *Nb*HIPP26 wild type and altered proteins S-Acyl^{M}, Prenyl^{M}, S-Acyl^{M} Prenyl^{M} and HMA^{M} were investigated (Fig. 5A-D). Expression of HMA^{M} gave a phenotype essentially the same as wild type (compare Fig. 5A with Fig. 3A). In comparison to the wild type GFP-HIPP26 and HMA^{M}, GFP fluorescence in the cells expressing each of the other three altered proteins was observed to accumulate more strongly in the nucleus (Fig. 5B-D). In addition, GFP labelling of the plasma membrane was reduced and substantial fluorescence was observed in the cytosol and in some motile vesicles. The phenotypes of the three altered proteins were similar, except that there were fewer motile vesicles and more cytosolic labelling in cells expressing the double modified protein, S-Acyl^{M} Prenyl^{M}. These experiments indicated that *Nb*HIPP26 binding to the plasma membrane and PD was weaker in the altered (non-lipidated) proteins, suggesting that disruption of the lipid anchors results in release of the proteins to the cytoplasm and allowing the subsequent marked increase in nuclear accumulation.

In order to study the association of the wild type and altered *Nb*HIPP26 proteins with the plasma membrane in more detail, cells expressing the GFP fusion proteins were plasmolysed to pull the plasma membrane away from the cell wall (Fig. 5E-H). It is known that as the protoplast retracts away from the wall, the plasma membrane is stretched into thin Hechtian strands where it appears as thin tubules with associated small blebs and vesicles along the strands. The wild-type *Nb*HIPP26 protein was associated with a profusion of these extra-protoplast structures (Fig. 5E). In cells expressing either Prenyl^{M} or S-Acyl^{M}, GFP fluorescence was associated with some membrane-derived structures external to the protoplast (e.g. Hechtian strands, vesicles and membrane blebs), which formed as the plasma membrane pulled off the cell wall as the protoplast retracted. The amount of apoplastic fluorescence was reduced compared to wild type (Fig. 5F and G), and expression of S-Acyl^{M} Prenyl^{M} showed almost no association with remnants of plasma membrane in the region between the protoplast and cell wall (Fig. 5H). These results suggest that lipidation is required to retain *Nb*HIPP26 in association with membranes supporting a mechanism whereby interaction with TGB1 disturbs or disrupts *Nb*HIPP26 lipidation, resulting in dissociation of the *Nb*HIPP26-TGB1 complex from the membranes at the cell periphery.

_Nb_HIPP26 is expressed specifically in the vascular parenchyma and induced by drought and PMTV infection

Analysis of the nucleotide sequence up to 2 kb upstream from the *NbHIPP26* ORF showed the presence of multiple stress responsive elements (Supplemental Fig. 2). A region of approx. 1 kb upstream of the predicted translational start site of *NbHIPP26* thought to encompass the promoter sequence was cloned and fused to the *uidA* gene, and the resulting construct used to transform *N. benthamiana* plants. Seven independent transgenic lines were tested for β-glucoronidase (GUS) expression and staining was visibly concentrated in the vascular tissue of all plants. In whole plants, GUS staining was seen predominantly in the vasculature of lower leaves, stems, and roots (Fig. 6A, B and C). At low magnification (Fig. 6D and E), GUS activity was seen in all vein classes of mature *N. benthamiana* leaves. Transverse sections of major veins (Fig. 6F and G) showed GUS activity to be concentrated in the periphery of cells within the vascular bundles. Vascular parenchyma cells (associated with both phloem and xylem tissue) showed blue staining, with the strongest promoter activity associated with clusters of phloem cells (Fig. 6F; areas outlined in red, and Fig. 6G). Phloem tissue was identified as clusters of cells containing small cells (sieve elements) that lie immediately adaxial and abaxial to the xylem tissue. These results are supported by experiments using $HIPP26_{pro}$*:uidA* constructs in Arabidopsis which also show strong vascular expression of GUS activity (Barth et al., 2009) and are consistent with previous reports that stress-related genes are preferentially expressed in the vascular tissues (reviewed in Turgeon and Wolf, 2009).

NbHIPP26 expression was measured in different tissues by reverse transcription quantitative PCR (RT-qPCR) and was shown to be most strongly expressed in root tissues, followed by the hypocotyl but was detected in all tissues sampled (Figure 6H).

To establish whether *NbHIPP26* is induced by drought, *N. benthamiana* plants were subjected to drought stress by removing whole plants from the growing medium and exposing them to air at room temperature for 30-240 min, during which *NbHIPP26* expression was measured by RT-qPCR. Relative *NbHIPP26* expression was compared against the endogenous genes *PP2A* and *F-BOX* and was shown to increase over time in response to drought stress (Fig. 7A).

To determine whether *NbHIPP26* is also induced by virus infection, gene expression levels were investigated in PMTV-infected *N. benthamiana*. Total RNA was obtained from upper non-inoculated leaves. Relative expression levels of *NbHIPP26* were compared by RT-qPCR against three endogenous genes (*PP2A*, *CDC2* and *F-BOX*) in both virus-infected and mock-

inoculated plants at different time points post inoculation. Relative *NbHIPP26* expression increased in approx. 71% of PMTV-infected plants tested between 10 and 14 days post inoculation (dpi) compared to mock inoculated plants (Fig. 7B shows a representative experiment at 12 dpi).

PMTV infection protects *N. benthamiana* from drought stress

Plants infected with some plant viruses are known to be drought tolerant (Xu et al., 2008). Therefore, we investigated whether infection with PMTV influenced drought tolerance. *N. benthamiana* plants were manually inoculated with PMTV or mock inoculated and then maintained in a glasshouse for 14 days to allow systemic spread. Water was then withheld, and plants were monitored for visible drought response. After a further 13 days, non-infected plants showed signs of wilting, but in marked contrast the infected plants did not wilt at this time point (cf. Fig. 8A and 8B). We then tested whether PMTV could protect plants from drought by withholding water until both mock- (Fig. 8C,D and E) and PMTV-infected plants (Fig. 8F,G and H) were completely wilted and then re-watering the plants. The results were similar in two independent experiments: the numbers of plants surviving (as judged by the presence of new green shoots)/number tested were: experiment one, 2/10 mock-infected, 5/8 PMTV-infected; experiment two, 2/5 mock-infected, 7/10 PMTV-infected. Thus fewer mock-inoculated plants survived, 27%, compared with plants infected with PMTV, 67%.

Knock down of Nb*HIPP26* expression inhibits PMTV long distance movement

To examine the role of HIPP26 in PMTV movement a tobacco rattle virus (TRV)-based system was used to knock down expression of *HIPP26* in *N. benthamiana* plants. A 120 bp fragment of *NbHIPP26* was cloned into TRV in an anti-sense orientation (TRV:HIPP26), and the results showed that expression of the targeted *HIPP26* gene was specifically reduced in TRV-RNA1 + TRV:HIPP26 inoculated plants compared to the empty vector controls (TRV-RNA1 + TRV:00) (Fig. 9A). The *NbHIPP26*-silenced and control plants were challenged with PMTV and analysed two weeks post inoculation. RT-qPCR of RNA samples isolated from the upper non-inoculated leaves of PMTV-inoculated plants revealed accumulation of all three genomic RNA components (Fig. 9B). However, accumulation of all three virus genomic components in upper leaves of the plants silenced for *NbHIPP26* was markedly decreased as compared to TRV:00 control plants (Fig. 9B). Quantitative analysis of PMTV accumulation by ELISA (to

detect virus particles) two weeks post inoculation revealed similar levels of PMTV accumulation in inoculated leaves of plants silenced for *NbHIPP26* and in non-silenced plants inoculated with TRV:00 (Fig. 9C), indicating that the cell-to-cell movement of virus particles and accumulation in inoculated leaves was not affected. However, in the upper leaves of plants silenced for *NbHIPP26*, PMTV particles accumulated in much lower amounts as compared to the control plants (absorbance values were ~30% of control; $P<0.05$ Student's two tailed *t* test; Fig. 9C). Seven of thirty-six (19%) *NbHIPP26*-silenced plants challenged with PMTV over six experiments were completely negative for the presence of virus particles in ELISA while all control plants were infected with PMTV.

As PMTV moves long distance both as virus particles and vRNPs, we next asked whether systemic movement of vRNPs was affected in the plants silenced for *NbHIPP26*. To address this question, *NbHIPP26*-silenced and control plants were inoculated with RNA-Rep and RNA-TGB *in vitro* generated transcripts and analysed two weeks post inoculation. RT-qPCR of RNA samples isolated from the upper non-inoculated leaves of plants challenged with these two genomic components revealed accumulation of RNA-Rep and RNA-TGB (Fig. 9D). However, accumulation of these two genomic components in upper leaves of the plants silenced for *NbHIPP26* was markedly decreased as compared to TRV:00 control plants ($P<0.05$; Fig. 9D). Hence, collectively, the results show that systemic movement of both virus particles and vRNPs but not cell-to-cell movement in *N. benthamiana* plants silenced for *NbHIPP26* was inhibited. Overall, the results show that the interaction of PMTV TGB1 with *Nb*HIPP26 is essential for efficient systemic movement of the virus.

DISCUSSION

TGB1 plays a central role in the long-distance movement of PMTV. TGB1 accumulates in the nucleolus, and we previously showed that nucleolar accumulation by an importin-α-dependent mechanism contributes to long distance movement (Lukhovitskaya et al., 2015). Here we further investigated the interactions of TGB1 with plant proteins and the role of TGB1 nucleolar accumulation in systemic virus movement. Y2H experiments showed that TGB1 interacted with the *N. benthamiana* homologue of the Arabidopsis protein HIPP26, and sequence analysis revealed a high level of sequence identity (84%) between the two proteins.

HIPP26 is a member of a family of proteins that contain heavy metal-binding and C-terminal isoprenylation motifs.

Previously, *Nb*HIPP26 was found to be present in the PD proteome (Fernandez-Calvino et al., 2011) and the plasma membrane proteome (Marmagne et al., 2007), and YFP-TGB1 accumulated in PD when expressed from a virus clone (Wright et al., 2010). HIPP proteins, like many membrane associated proteins, are modified post-translationally by the attachment of lipid moieties. HIPP26 contains a C-terminal motif, CVVM, identical to the known prenylation motif of N-Ras, and we showed that *Nb*HIPP26 is lipid-modified by S-acylation through residue C^{13}. Both modifications were shown to be important for the observed localisation of *Nb*HIPP26 at PD and the plasma membrane. Mutation of both S-acylation and CVVM lipidation sites led to a marked accumulation of HIPP26 in the nucleus and nucleolus and loss from the cell periphery. S-acylation provides a membrane association strength many times greater than a prenyl group (more akin to a transmembrane domain) but, critically, is reversible. This provides a mechanism to change the membrane association of proteins, as has been shown for active and inactive type-I ROP GTPases (Sorek et al., 2017). While lipid modifications usually promote protein-membrane interaction, they can also mediate protein-protein interactions as illustrated by the prenyl group-mediated interaction of K-Ras with Galectin-1 and -3 (Ashery et al., 2006). Given that TGB1 binding to *Nb*HIPP26 requires an intact prenylation site, it is possible that TGB1 binds *Nb*HIPP26 at least partly through the prenyl moiety. This may mask the ability of the prenyl group to interact with the membrane and promote dissociation of *Nb*HIPP26 from the plasma membrane environment. Consistent with this idea, BiFC showed that on interaction with TGB1, the *Nb*HIPP26-TGB1 complex localised predominantly to microtubules and the nucleolus with a small quantity in the nucleoplasm. The following model is consistent with our data; TGB1 binds *Nb*HIPP26, most likely at PD, through the *Nb*HIPP26 prenyl group, leading to *Nb*HIPP26 conformational change and exposure of the S-acyl thioester. The thioester is now accessible for cleavage and releases *Nb*HIPP26 from the plasma membrane, allowing trafficking of the *Nb*HIPP26-TGB1 complex to the nucleus via microtubules.

N. benthamiana plants expressing HIPP26 promoter-reporter fusions revealed vascular tissue specific expression similar to that found for *At*HIPP26, suggesting a similar role for *Nb*HIPP26 in drought tolerance. In addition, PMTV infection helps to protect *N. benthamiana* plants against drought, presumably due to the increased expression of *Nb*HIPP26 during the systemic infection process.

VIGS of *NbHIPP26* expression resulted in decreased accumulation of PMTV in upper (non-inoculated) leaves. In many of the *NbHIPP26* knock down plants there was approx. 50% less virus than in non-inoculated plants, and in 19% of plants no particles were detected whereas particles were detected in all of the non-silenced control plants.

The eukaryotic cell nucleolus contains the factors needed for its main function in ribosome subunit production, and this is where ribosomal RNA transcription, processing, and ribosomal RNP assembly occur. In mammalian cells, major changes occur in the organisation and protein composition of the nucleolus in response to stress where it plays a key role in responding to abiotic and biotic stress signalling (Boulon et al., 2010). Several positive-strand RNA viruses encode proteins that localize to the nucleolus (Taliansky et al., 2010), including the GRV ORF3 movement protein. The GRV ORF3 movement protein enters the nucleolus and associates with the nucleolar protein fibrillarin before the fibrillarin-ORF3 complex returns to the cytoplasm, enabling the formation of vRNP needed for long distance movement (Kim et al., 2007).

The TMV replication protein interacts with three vascular-expressed auxin/indole acetic acid (Aux/IAA) transcriptional regulators, including IAA26, whichis expressed in phloem companion cells (Padmanabhan et al., 2006). These Aux/IAA proteins are thought to function in regulation of genes involved in phloem loading. Recently, it has been shown that TMV infection inhibits nuclear localisation of Aux/IAA proteins, leading to transcriptional reprogramming of mature vascular tissue, which correlates with enhanced TMV movement and spread in the phloem of older leaves (Collum et al., 2016).

Our results indicate that the nuclear and nucleolar association of PMTV TGB1 has a different function in virus long-distance movement to that described for other viruses. They suggest that nuclear association of plant RNA virus components can manipulate transcriptional regulation and re-programme gene expression in vascular tissue and that this may be a more general strategy used by plant viruses to facilitate long-distance movement than previously supposed (Solovyev and Savenkov, 2014; Collum et al., 2016).

However, another consideration is whether the interaction of TGB1 with *Nb*HIPP26 may have a role in pathogenicity. As stated before, HIPPs are a large family of plant proteins that play roles in response to both abiotic and biotic stresses (de Abreu-Neto et al., 2013). For example, the cytoplasmically-located rice protein *Os*HIPP05 is a recessive susceptibility factor in rice blast disease; the resistant allele carries deletions in proline-rich motifs thought to be targeted by pathogen protein(s) to facilitate infection (Fukuoka et al., 2009). While we cannot

completely rule out that the interaction of *Nb*HIPP26 with PMTV-TGB1 may be suppressing a putative role of HIPP26 in host defence (e.g. by transporting HIPP26 on microtubules for degradation), we think that this is unlikely since, logically, knock down of *Nb*HIPP26 would result in increased spread of infection and not inhibition as we observed.

Our results showed that *Nb*HIPP26 moves as a complex with TGB1 and accumulates in the nucleus. By analogy to *At*HIPP26, which interacts in the nucleus with the transcriptional activator ZFHD1 (Tran et al., 2004, 2007; Barth et al., 2009), we suggest that the *N.benthamiana* TGB1 interaction leads to the promotion of drought tolerance by activating expression of dehydration-inducible genes. Loss of function of HIPP26 in an Arabidopsis mutant inhibits expression of stress-responsive genes regulated by ZFHD1, and Barth et al. (2009) proposes a mechanism where the heavy metal carried by HIPP26 inactivates or displaces the ZF-HD repressor and allows activation of the stress-inducible genes. Our data suggests a model where once the PMTV infection reaches the vascular tissue, TGB1 binds to *Nb*HIPP26. The *Nb*HIPP26/TGB1 complex is then transferred from the plasma membrane or PDs via microtubule-directed transfer to the nucleus and nucleolus where HIPP26 accumulation leads to upregulation of dehydration-responsive gene expression in the vasculature and establishment of a drought-tolerant state in the plant. The changes in vascular gene expression also allow virions or RNPs to enter the sieve element/companion cell complex for long distance movement (Fig. 10). The PMTV genome is tripartite, and intact virus particles are essential for vector transmission (Cowan et al., 1997; Reavy et al., 1998). A mechanism allowing systemic movement of virions or RNPs (including all genome components) ensures genome integrity for successful future vector-based transmission of fully infective virus from tissues far removed from the initial infection site.

MATERIALS AND METHODS

Yeast Two-Hybrid Analysis

Yeast two-hybrid screening was performed by Hybrigenics Services, S.A.S., Paris, France (http://www.hybrigenics-services.com). The Hybrigenics system employs plasmids pB27 (containing the LexA binding domain; BD) and pP6 (containing the P6 activation domain; AD) derived from the original pBTM116 (Vojtek and Hollenberg, 1995) and pGADGH (Bartel et

al., 1993) plasmids, respectively. The coding sequence for the full-length protein TGB1 (GenBank accession number AJ277556) was PCR-amplified and cloned into pB27 as a C-terminal fusion to LexA. The construct was confirmed by sequencing the entire insert and used as a bait to screen a randomly-primed tobacco (*Nicotiana benthamiana*) cDNA library constructed into pP6. One hundred twenty-five million clones (12-fold the complexity of the library) were screened using a mating approach with YHGX13 (Y187 ade2-101::loxP-kanMX-loxP, mata) and L40ΔGal4 (mata) yeast strains as previously described (Fromont-Racine et al., 1997). One hundred eighty-six colonies were selected on a medium lacking tryptophan, leucine, and histidine. The prey fragments of the positive clones were amplified by PCR and sequenced at their 5' and 3' junctions. The resulting sequences were used to identify the corresponding interacting proteins in the GenBank database (NCBI) using a fully automated procedure.

A confidence score (PBS, for Predicted Biological Score) was attributed to each interaction as previously described (Formstecher et al., 2005). The PBS relies on two different levels of analysis. Firstly, a local score takes into account the redundancy and independence of prey fragments, as well as the distribution of reading frames and stop codons in overlapping fragments. Secondly, a global score takes into account the interactions found in all the screens performed at Hybrigenics using the same library. This global score represents the probability of an interaction being nonspecific. For practical use, the scores were divided into four categories, from A (highest confidence) to D (lowest confidence). A fifth category (E) specifically flags interactions involving highly connected prey domains previously found several times in screens performed on libraries derived from the same organism. Finally, several of these highly connected domains were confirmed as false-positives of the technique and were tagged as F. The PBS scores have been shown to positively correlate with the biological significance of interactions (Rain et al., 2001; Wojcik et al., 2002).

Plasmids of representative interacting clone pP6-A44 were obtained from Hybrigenics and tested again at the James Hutton Institute. The interaction between Gal4 and LexA protein fusions was examined in the yeast L40 strain [MATa his3D200 trp1-901 leu 2-3,112 ade2 lys2-801am URA3::(lexAop)8-lacZ, LYS2::(lexAop)4-HIS3]. Yeast cells were co-transformed by the small-scale lithium acetate yeast transformation method as described in Clontech's Yeast Protocols Handbook. Transformants were selected on synthetic dropout minimal medium base containing 2% (w/v) glucose and dropout supplements lacking Leu and Trp (+H) or lacking Leu, Trp, and His (-H). In experiments with LexA-IMP and LexA-PVXTGB1, a small amount

of autoactivation was observed, which was suppressed by the addition of 10 and 50 mM 3-AT, respectively.

Primer sequences used for cloning are given in the Supplemental Table. The coding sequence for the NbImportin-α 1 (ABMO5487; GenBank Accession EF137253.1) was PCR amplified using primers IMPFOR and IMPREV (Lukhovitskaya et al., 2015) with template plasmid eGFP-NbIMP-α1 and cloned into pP6 (Hybrigenics). The virus movement proteins were cloned into the bait vector pB27. PVX TGB1 was PCR amplified from pPVX201 (Baulcombe et al., 1995) using primers PVXTGB1FOR and PVXTGB1REV, TMV 30K was PCR amplified from TMV-30B (Shivprasad et al., 1999) using primers TMV30KFOR and TMV30KREV, and BSMV TGB1 was PCR amplified from a BSMV RNA β cDNA clone (Torrance et al., 2006) using primers BSMVTGB1FOR and BSMVTGB1REV.

Sequence alignments and phylogenies

To place the clone A44 (*Nb*HIPP26) within the full family repertoire of HIPP proteins previously identified in Arabidopsis (*Arabidopsis thaliana*), the full length protein sequences of 45 HIPP proteins identified by de Abreu-Neto (de Abreu-Neto et al., 2013) were downloaded from GenBank (Release 205)(Benson et al., 2013). A multiple alignment of these 45 proteins along with the clone sequence A44 was made using MUSCLE with default parameters (Edgar, 2004) and a phylogeny was created using the neighbour joining method within MEGA (v6.06). The phylogenetic tree was rendered as a mid-point rooted tree with proportional branch lengths using FigureTree (v1.4.2) (Rambault, 2012).

To assess the percentage sequence similarity between the *Nb*HIPP26 protein and its homologues in potato (*Solanum tuberosum*), tomato (*Solanum lycopersicum*) and Arabidopsis, a multiple sequence alignment was made using MUSCLE with default parameters (Edgar, 2004). A sequence identity matrix of protein-protein pairs was calculated from the alignment.

Plant material

N. benthamiana plants were grown in a glasshouse (21°C day and 16°C night; 18 h day length with supplementary lighting of 250 W m^{-2}). *N. benthamiana* plants expressing mRFP:AtFib1 fusions (RFP-labelled fibrillarin) were given by Michael Goodin. *N. benthamiana* plants expressing mOrange-LTi6b (mOrange labelled plasma membrane) were created at the James Hutton Institute (Wright et al., 2017).

To check gene expression: *N. benthamiana* plants (35 days post germination) were removed from growth medium. The roots were rinsed free of compost and plants left in air on filter paper for the designated time. Tissues were then processed for RT-qPCR as described below.

To test the effect of PMTV infection: In two independent experiments, *N. benthamiana* plants were mechanically inoculated with PMTV or water (mock) and maintained in the glasshouse (as described above). The upper non-inoculated leaves were tested by ELISA 12-14 days post inoculation (dpi) to confirm systemic infection. Water was then withheld until the plants reached wilting point, and then watering was restored.

Cloning of plasmids and mutants

All of the plasmids were constructed so that fluorescent protein expression was driven by the 35S promoter. Primer sequences are provided in the Supplemental Table.

Constructs used for confocal microscopy: The *Nb*HIPP26 encoding sequence was amplified from *N. benthamiana* total RNA using attB adapter flanked primers HIPP26FOR and HIPP26REV and recombined into pDONR207 using Gateway BP Clonase II. This entry clone was recombined with pB7WGF2 (Karimi et al., 2002) using Gateway LR Clonase II to create plasmid eGFP-HIPP26.

Constructs used for BiFC: pDONR207 entry clone constructs containing the sequences coding for *Nb*HIPP26 and TGB1 were recombined into pSITE-cEYFP-N1 (Martin et al., 2009) using Gateway LR Clonase II. pDONR207 constructs containing the sequences coding for TGB1, *Nb*HIPP26^{C174G}, and TGB1Δ84 were recombined into pSITE-nEYFP-C1 (Martin et al., 2009) using Gateway LR Clonase II (Invitrogen).

Construction of HIPP 26 mutants: Mutations in the *NbHIPP26* sequence were made using the QuikChange Site-Directed Mutagenesis kit (Stratagene). The Cys^{152} codon was changed to GGA (glycine codon) using primers CVVMFOR and CVVMREV to create *Nb*HIPP26^{C152G}. The cysteine^{38} and cysteine^{41} codons within the HMA domain were changed to GGA using primers HMAmutFOR and HMAmutREV to create *Nb*HIPP26^{G38C,G41C}. The Cys^{13} codon was mutated to GGA using primers HIPP26cysmutFOR and HIPP26cysmutREV to create *Nb*HIPP26^{C13G}. A double mutant was created where the Cys^{13} and Cys^{152} codons were both mutated to GGA using primers HIPP26cysmutFOR and HIPP26cysmutREV with template GFP-*Nb*HIPP26^{C152G} .

Live cell imaging of fluorescent proteins

Unless otherwise stated, all constructs were delivered into *N. benthamiana* leaves by agro-infiltration through the abaxial stomata. *Agrobacterium tumefaciens* (strain LB4404) cultures carrying the plasmid constructs used for live cell imaging were grown overnight (16 h) at 28ºC in Luria Bertani LB media supplemented with Rifampicin (50 mg/L) and Spectinomycin (100 mg/L). Cells were collected by centrifugation at 3500 rpm for 15 min and then resuspended in infiltration buffer (10 mM $MgCl_2$, 10 mM MES, 150 µM Acetosyringone) to a final $O.D._{600}$ of 0.1 before infiltration into the abaxial side of *N. benthamiana* leaves. All confocal imaging was conducted using a Leica TCS-SP2 AOBS spectral confocal scanner (Leica Microsystems GmbH, Heidelberg, Germany). Unless otherwise stated, images were obtained using a Leica HCX APO x63/0.9W water-dipping lens and whole lesions using a HCX PL Fluotar ×1.6/0.05 lens. GFP and YFP were imaged sequentially: GFP excitation at 488 nm, emission at 490 to 510 nm; YFP excitation at 514 nm, emission at 535 to 545 nm. GFP and mRFP were imaged sequentially: GFP excitation at 488 nm, emission at 500 to 530 nm; mRFP excitation at 561 nm, emission at 590 to 630 nm. Images of GUS localisation were taken on a Zeiss AxioImager microscope (Carl Zeiss Ltd, Cambridge, UK) equipped with a Zeiss Axiocam HRC camera. Images were prepared using Adobe Photoshop CS5.

Reverse transcription quantitative real-time polymerase chain reaction (RT-qPCR)

Five-week-old seedlings of *N. benthamiana* were manually inoculated with leaf extracts from PMTV-infected *N. benthamiana* or mock-inoculated plants and grown in a Snijders growth cabinet providing a 12 h day (with a light intensity setting of 375 µmol m^{-2} s^{-1}), 12 h night at constant 14ºC. Total RNA was isolated from leaf samples using the RNeasy Plant Minikit with the on-column DNaseI treatment for removal of genomic DNA contamination according to the manufacturer's protocol (Qiagen). Reverse transcription of 5µg of total RNA was performed using Ready-To-Go-You-Prime-First Strand Beads (GE Healthcare) according to the manufacturer's protocol. cDNA was used as a template for real-time PCR using the Universal Probe Library System ((https://www.roche-applied-science.com/sis/rtpcr/upl/index.jsp). Reactions were performed in 25 µl containing 1 x FastStart TaqMan® Probe Master (supplemented with ROX reference dye). Gene-specific primers and probes were used at a concentration of 0.2 µM and 0.1 µM, respectively. Thermal cycling conditions were 95 °C for 10 min followed by 40 cycles (15 s at 94 °C, 60 s at 60 °C). Relative expression levels were

calculated and the primers validated using the ΔΔCt method (Livak, 1997) using data obtained from the reference controls: *PROTEIN PHOSPHATASE 2A* (*PP2A*, Accession TC21939), *F-Box protein* (*F-BOX*, Accession Niben.v0.3.Ctg24993647), *CYCLIN-DEPENDENT KINASE 2* (*CDC2*, Accession Q40482). The Universal ProbeLibrary (UPL) primer pairs and probe sequences were as follows: cdc2FOR/cdc2REV with UPL probe number 19; F-BOXFOR/F-BOXREV with UPL probe number 1; PP2AFOR/PP2AREV with UPL probe number 22; HIPP26QFOR/HIPP26QREV with UPL probe number 112. Primer sequences are provided in the Supplemental Table.

Tobacco rattle virus-mediated VIGS

To facilitate the use of tobacco rattle virus (TRV)-mediated VIGS, the 0.12 kb fragment of *Nb*HIPP26 cDNA was cloned into TRV:00 (TRV RNA2 infectious cDNA clone) in an anti-sense orientation. Two weeks post TRV-RNA1 + TRV:*HIPP26* infection, reverse transcription (RT)-PCR tests were carried out on upper (non-inoculated infected) leaves using primers designed to amplify endogenous *Nb*HIPP26RNA transcripts.

Agrobacterium tumefaciens (strain C58C1) cultures carrying the TRV-RNA1 plasmid and TRV-RNA2 cassettes were grown overnight, collected by centrifugation at 3500 rpm for 10 min, and then resuspended in infiltration buffer to a final $O.D._{600}$ of 0.5. Equal volumes of TRV-RNA2-expressing cells and the TRV-RNA1 cultures were mixed and infiltrated into the abaxial side of two *N. benthamiana* leaves on seedlings at the four-leaf stage. Plants were grown for two weeks prior to challenging with PMTV. The uppermost leaves were collected and assayed for *Nb*HIPP26 silencing by RT-qPCR.

RT-qPCR for PMTV: Results were obtained from two RT-qPCR experiments (three plants per treatment, either TRV:HIPP26 or TRV:00). Reverse transcription of 1µg of total RNA was performed using an iScript cDNA synthesis kit (Bio-Rad) according to the manufacturer's instructions. Four µl of ten-fold diluted cDNA was used as a template for real-time PCR. Reactions were performed in a 20 µl reaction containing 2x DyNAmo Flash SYBR Green master mix supplemented with ROX passive reference dye, along with 0.2 µM primers specific to the respective gene of interest. Data analysis was performed using the Bio-Rad CFX manager 3.1 (Bio-Rad).

In order to obtain a standard curve, ten-fold serial dilutions for respective plasmids of RNA-Rep, RNA-CP, and RNA-TGB were made from 100 pg/µl to 0.01 pg/µl. Four µl of the diluted plasmid was used as a template for the real-time PCR. Total number of viral copies was calculated using the formula of "No. of molecules = (ng of dsDNA) x (no. of molecules/mole) x (1/Number of bases) x (1/660 g) x (1/10^9 ng/g)". The Avogadro constant of 6,023 x 10^23 molecules/mole was used to calculate the number of copies, and the formula weight of base pairs in dsDNA was considered as 660 g. See also copy number calculator for real-time PCR: http://scienceprimer.com/copy-number-calculator-for-realtime-pcr.

S-acylation assays

Protein S-acylation state was assessed as described by Hemsley et al., 2008 and Forrester et al., 2011 with some modifications. GFP-tagged *Nb*HIPP26 constructs were transiently expressed in *N. benthamiana* using agro-infiltration along with the p19 silencing suppressor (Voinnet et al., 2003) at $O.D._{600}$ of 0.1 for both. Flash frozen tissue was ground in liquid nitrogen to a fine powder and solubilised in four volumes of TENS buffer (100 mM Tris.HCl pH7.2, 5 mM EDTA, 150 mM NaCl, 2.5% SDS) with protease inhibitors (Sigma P9599, 5 ul ml^{-1}) and 25 mM N-ethylmaleimide (NEM) at 20°C for 10 minutes with constant mixing. Lysates were clarified for 5 minutes at 3000 x **g**, passed through two layers of miracloth, and re-centrifuged at 16000 x **g** for 10 minutes, all at 20°C. The clarified supernatant was retained, and protein concentration was calculated using a BCA assay. One mg of protein was diluted to 1 ml final volume in TENS buffer with protease inhibitors and 25 mM NEM and incubated with constant mixing for 1 hour at 20°C. Samples were precipitated with chloroform/methanol (Wessel and Flugge, 1984), briefly air dried, and resuspended in 1 ml (100 mM Tris.HCl pH7.2, 5 mM EDTA, 150 mM NaCl, 6M Urea, 2% SDS) with heating to 37°C and gentle mixing. Samples were then split into two 0.5-ml aliquots in 1.5-ml microfuge tubes. One aliquot was treated with 0.5 ml of 1M hydroxylamine.HCl (pH7.2 with 10M NaOH, made fresh) while the other (negative control) was treated with 0.5 ml of 1M NaCl. After brief mixing, 100 µl was removed from each as a loading control and incubated at 20°C for 1 hour before precipitation with chloroform/methanol. To the remainder, 40 µl of 50% thiopropyl sepharose 6B bead suspension in TENS buffer was added to capture S-acylated proteins and gently mixed at 20°C for 1 hour. Beads were washed 3x with 1 ml TENS buffer for 5 minutes each then dried by aspiration. Loading controls and bead samples were resuspended in a 2x SDS-PAGE sample

buffer containing 6M urea and 50 µl ml-1 β-mercaptoethanol and heated at 37°C for 30 minutes with frequent mixing. Samples were subsequently run on 12.5% Laemli SDS-PAGE gels and blotted to a PVDF membrane. EGFP-HIP26 variants were detected using an anti-GFP monoclonal antibody (Abgent AM1009a). Blots were imaged using GeneSys on a G:BOX XT4 GelDoc system and quantified using GeneTools (Syngene).

Preparation of *Nb*HIPP26 promoter construct and production of transgenic plants

The 1128 bp region upstream of the translational start site of *NbHIPP26* gene was amplified using primers HIPProFOR and HIPProREV on genomic DNA of *N. benthamiana* (Nb-1). The resulting PCR fragment was cloned between *Sac*II-*Xba*I sites of a binary vector, pORE-R2 (Coutu *et al*., 2007), in front of the *uidA* gene. The resulting vector was named pORE-R2*Nb*HIPP26. This vector was transferred to *Agrobacterium tumefaciens* (strain AGL1). Wild-type *N. benthamiana* (Sainsbury) plants were transformed, and transgenic plants were regenerated as described by Clemente (2006).

GUS-staining of transgenic plants.

The *HIPP26*$_{pro}$*:uidA* transgenic *N. benthamiana* were grown in a glasshouse (21°C day and 16 °C night; 18 h day length with supplementary lighting of 250 W m^{-2}). Samples of leaf and petiole were taken from plants 10 weeks post germination and embedded in 3% agar (Oxoid No.1; Oxoid Ltd, Basingstoke, UK) to allow suitable orientation in a further block of agar mounted on a vibroslice (Model 752M, Campden Instruments Ltd., Loughborough, UK) to allow transverse sections to be cut through major veins in the leaf lamina. Sections of fresh tissue were cut approximately 200 µm thick and dropped immediately into GUS assay buffer (28 mM NaH_2PO_4, 72 mM Na_2HPO_4 [pH 7.2] containing 0.2 % w.v. Triton X-100, 2 mM each of potassium hexacyanoferrate(III) and potassium hexacyanoferrate(III) trihydrate) before being vacuum infiltrated and incubated overnight in the dark at 37°C. Sections were subsequently washed in several changes of 70% ethanol and stored in 70% ethanol before being mounted in water and imaged using a Leica DMFLS light microscope. Photographs were collected using a Zeiss Axiocam to show the distribution of GUS stain.

Accession Numbers

Arabidopsis HIPP26-At4g38580 **N Benth HIPP26** Niben101Scf02621g04026.1

Supplemental Data

Supplemental Fig 1. Arabidopsis HIPP family phylogeny.

Supplemental Fig 2. Cis regulatory elements upstream of the HIPP26 ORF.

Supplemental Table. List of primers and sequences.

Supplemental Movie S1. Motile vesicles tagged with GFP-HIPP26.

ACKNOWLEDGEMENTS

We thank Michael Goodin for the RFP-labelled fibrillarin plants and Indu Sama and Nina Lukovitskaya for technical assistance with VIGS.

Figure legends

Figure 1. HIPP phylogeny. A, Diagrammatic representation of the phylogenetic relationship between A44 and five HIPP families (de Abreu-Neto et al., 2013). A44 is related to Arabidopsis HIPP sub family II and most closely related to HIPP26 (for full phylogeny see Supplementary Fig. 1). B, Multiple alignments of HIPP26 proteins from *Arabidopsis thaliana* (Arath), *Nicotiana benthamiana* (Benth), *Solanum lycopersicum* (Tomato) and *Solanum tuberosum* (Potato) with A44. The alignments were made using MUSCLE with default parameters (Edgar, 2004). The line below the alignment marks conserved positions: (*) single, fully conserved residue; (:) 'strong' group, fully conserved, scoring >0.5 in the Gonnet PAM 250 matrix; (.) 'weaker' group, fully conserved, scoring ≤0.5 in the Gonnet PAM 250 matrix. C, Percentage sequence identities for the four HIPP26 proteins and A44 shown in the alignment in Fig. 1B.

Figure 2. Yeast two-hybrid interaction analysis. A, Virus movement proteins, potato mop-top virus (PMTV) TGB1, beet virus Q (BVQ) TGB1, tobacco mosaic virus (TMV) 30K, potato

virus X (PVX) TGB1, and barley stripe mosaic virus (BSMV) TGB1 proteins were fused to the Lex A binding domain (BD) and tested for interaction with HIPP26 fused to the p6 activation domain (AD) on double drop out (+H) or triple drop out (-H) media. B, Diagrammatic representation of wild type and mutants in PMTV TGB1 (Δ84 and Δ149 deleted by aa 1-84 and aa 1-149, respectively) and wild type and mutants of HIPP26 showing the position of the cysteine residues changed to glycine in putative prenylation ($Prenyl^{M}$), S-Acylation ($C^{13}G$), and heavy metal-associated (HMA^{M}) domains. C, TGB1 wild type and deletion mutants were tested for interaction with HIPP26, $Prenyl^{M}$, and HMA^{M}. D, Amino acid sequence alignment of PMTV and BVQ N-terminal amino acids.

Figure 3. Sub-cellular localisations of HIPP26 and the HIPP26-TGB1 complex. A, The GFP-HIPP26 fusion protein localised to the plasma membrane and a population of small (~2 µm diameter), motile vesicles (arrowhead). A small amount of cytosolic labelling and faint transvacuolar strands were seen in some cells. GFP-HIPP26 was also present in the nucleus, both in the nucleoplasm and nucleolus, and to levels greater than would be expected as a result of passive diffusion from the low levels in the cytosol. HIPP26 was therefore targeted to the nucleus. B, Co-expression with an mRFP-fibrillarin (a nucleolar marker) showed precise co-localisation of GFP-HIPP26 in the nucleolus (indicated by arrowhead), while (C) co-expression and co-localisation with mOrange-LTi6b (a plasma membrane marker) showed that HIPP26 was also targeted to the plasma membrane. In addition to the motile vesicles that were seen in the cytoplasm of the cell periphery (cf. A and the Supplemental Movie), small non-motile punctate were also observed at the cell periphery (D). Co-localisation of these structures with an mRFP-tagged TMV 30K protein (a plasmodesmatal marker) showed that HIPP26 was also associated with a population of plasmodesmata (indicated by arrowheads).

E,F, Bimolecular fluorescence complementation (BiFC) analysis of PMTV TGB1 and *Nb*HIPP26 in *Nicotiana benthamiana* epidermal cells showed co-expression of Y-HIPP26 and TGB1-FP and re-constitution of yellow fluorescence localized to microtubules (E), to the nucleoplasm, and strong accumulation in the nucleolus (F). G, Co-expression of Y-HIPP26 and Δ84TGB1-FP showed re-constitution of yellow fluorescence localized to the nucleoplasm and strong accumulation in the nucleolus with no microtubule labelling. No complementation was obtained in control experiments where Y-HIPP26 or TGB1-FP were co-expressed with the complementary empty spilt YFP plasmid (no fluorescence was visible so no images are shown). H,I,J, Co-localisation of GFP-HIPP26 and mRFP-TGB1 (H, GFP; I, RFP and J,

merged) showed that both proteins localized to the nucleoplasm, nucleolus, and microtubules as expected from previously published data and the BiFC results. K, Expression of GFP-HIPP26 in PMTV-infected cell showed GFP fluorescence accumulated on microtubules (indicated by arrow heads). Scale bars: A and D = 10 µm, B = 5 µm, C = 10 µm for left panel of pairs and 5 µm for right panel of pairs, E = 10 µm, F = 5 µm, G = 10 µm, and J = 10 µm (used for H-J), and K = 5 µm

Figure 4. HIPP26 S-acylation. A, HIPP26 was S-acylated under normal plant growth conditions at Cys13. S-acylation was determined by the Acyl-RAC assay. Free cysteine residues were blocked with N-ethylmaleimide. S-acyl groups on cysteine residues within proteins were cleaved by hydroxylamine (Hyd +) to reveal free sulfhydryls. Sulfhydryl reactive thiopropyl Sepharose beads were used to capture free sulfhydryl-containing proteins. Negative controls lacked hydroxylamine (Hyd -). Signal strength in experimental lanes (EX Hyd+) showed the level of S-acylation. Loading controls (LC) demonstrated equal loading of hydroxylamine-treated and -untreated samples onto thiopropyl Sepharose beads. B. Relative quantification of blots shown in A. Mutation of the C-terminal CVVM motif cysteine ($Prenyl^{M}$) had a small effect on the S-acylation state (n = 2, *P*= 0.103, Student's one-tailed t-test) while changing Cys13 to Gly effectively abolished S-acylation (n = 2, *P* = 0.032). Values were calculated as intensity in EX Hyd+ lanes divided by intensity in LC Hyd+ lanes. Values are means represented as the proportion of wild type HIPP26 S-acylation. Error bars indicate ±SD.

Figure 5. Sub-cellular localisation of GFP-tagged *Nb*HIPP26 modified proteins. Four mutants expressing proteins with altered HMA (HMA^{M}) or lipidation domains ($S\text{-}Acyl^{M}$, $Prenyl^{M}$, and $S\text{-}Acyl^{M}$ $Prenyl^{M}$) were investigated (A-D, respectively). The HMA altered protein (A) was broadly similar to the wild-type HIPP26 (cf. Fig. 3A), showing plasma membrane labelling, low levels of cytosolic labelling, some small motile vesicle labeling, and accumulation in the nucleoplasm and nucleolus. In comparison, all three proteins altered in lipidation domains (B, C, and D) showed a significant increase in their nuclear accumulation (in both the nucleoli and nucleoplasm) and a reduction in the amount of plasma membrane labelling accompanied by an increase in the level of cytosolic fluorescence (seen most clearly in transvacuolar strands; arrows). Motile vesicles were seen in all three lipidation-deficient proteins, and the phenotypes were broadly similar, although cells expressing the doubly modified protein, $S\text{-}Acyl^{M}$ $Prenyl^{M}$ (D), showed fewer vesicles and more cytosolic localisation. Plasmolysis of cells expressing wild-type HIPP26 (WT; E) and the three altered

lipidation domainproteins (F-H) were also investigated. Here, the fluorescent cytosol was contained within the protoplast which retracted from the cell walls, leaving a black space in the extracellular space/apoplast. Arrowheads situated in the extracellular space point to the plasma membrane of the retracted protoplasts. Hechtian strands (stretched extensions of plasma membrane that extend from the plasmolysed protoplast to the cell wall), membrane blebs, and vesicles were clearly visible in the apoplast of the WT (arrows). These structures were not easily detected in bright-field images, but GFP fluorescence associated with the remaining membrane made them clearly visible in the confocal micrographs shown here. There were fewer of these structures and their morphology was somewhat altered in the cells expressing the proteins altered in a single lipid domain (F,G; arrows) and much less apparent in H, suggesting that removal of both lipidation domains reduced association with the plasma membrane in an additive way. Scale bars: A-H = 10 µm.

Figure 6. Images of glucuronidase (GUS) expression in *Nicotiana benthamiana* transformed with the *NbHIPP26* predicted promoter sequences fused to the *uidA* gene. GUS staining in mature plants (A), mature leaves (B), and roots (C)was detected throughout the vasculature of the mature plant but was not present in young sink leaves (A). Expression was particularly strong in the roots and hypocotyl, and in leaves, the GUS staining was more evident in older leaves (A – C). Expression in leaf veins appeared to follow the sink-source transition; GUS staining was evident in tip (source) veins while the basal (sink) portion was clearer (B). When studied in more detail in leaf veins, GUS was specifically localised to cells within the vascular bundle of all vein classes in mature leaves (D and E; arrows point to major vein classes and arrowheads to minor veins). At low magnification (D and E), there appeared to be little or no GUS staining in ground tissues between the veins, and intact leaf petioles did not show staining. Once cut however, GUS was visible in the vascular bundles of petioles. Images D and E show photographs of intact, mature *N. benthamiana* leaves photographed using a combination of bottom and top-lighting. F and G show GUS staining in transverse sections through major veins. (F and G), GUS staining localised to the periphery of many cells throughout the The vascular bundle (F) is delineated with a dotted black line, and phloem regions are outlined with red dotted lines. Some GUS staining was detected in cells surrounding the vascular bundles, but much less than inside the vascular system. (G) shows an enlargement of the phloem bundle marked in F with a red arrow. Blue GUS staining can clearly be seen in Xylem parenchyma (XP), phloem (P) cells, but is generally absent from metaxylem vessels (X), and bundle sheath (BS) are shown. Scale bars: A = 2 mm, B = 500 µm, C = 50 µm and D = 20 µm. (H) Relative

*NbHIPP26 e*xpression in different tissues measured by reverse transcription quantitative real-time PCR. The histogram bars show mean values ±SD (n=3, two independent experiments).

Figure 7. Relative expression of *NbHIPP26* in leaves of *N. benthamiana*. (A) Relative expression in *N. benthamiana* leaves after 30-240 min of drought stress (representative experiment showing means ±SD for each time point; n = 3) and (B) in PMTV-infected leaves 12 days post inoculation (representative experiment showing means ±SD for each time point; n = 8); showing that both drought stress and PMTV infection up-regulate *NbHIPP26* expression.

Figure 8. Representative images of PMTV- or mock-inoculated *N. benthamiana* plants after drought stress. (A) Mock- or (B) PMTV-inoculated plants after water was withheld for 13 days showing that PMTV-infected plants are more resilient to wilting compared with mock. Representative mock- (C,D,E) and PMTV-inoculated plants (F,G,H) before treatment (C,F), after withholding water (D,G), and after re-watering (E,H).; showing that PMTV-infected plants have produced green shoots and show recovery from drought treatment in contrast to the mock-inoculated plants (E).

Figure 9. Effects of the knock down of *NbHIPP26* expression on PMTV accumulation in upper non-inoculated leaves. A, Gene silencing of *NbHIPP26* gene in TRV:*HIPP26*-infected plants. Total RNAs were extracted from the upper leaves 14 days post infiltration (dpi) and were used for RT-qPCR. Data are means ± SD from three experiments (n =14 for each treatment). *NbF-BOX* and *NbPP2A* were used as normalization controls. B, Effect of *HIPP26* gene silencing on viral RNA levels in upper non-inoculated leaves. Total RNAs were extracted from the upper leaves 14 dpi with PMTV and used for absolute RT-qPCR. Ten-fold serial dilutions of full-length infectious cDNA clones of RNA-Rep, RNA-CP, and RNA-TGB were used to obtain standard curves and determine viral RNA copy number. *NbF-BOX* and *NbPP2A* were used as normalization controls. Data are means ±SD from four independent experiments with three or four biological replicates each. Asterisks indicate statistically significant differences compared with TRV:*00* (*t* test, $^{**}P<0.05$). C, ELISA was used to determine PMTV infection in inoculated and upper non-inoculated leaves of treated plants. Aabsorbance values (indicateding presence of PMTV capsid protein.) in samples of inoculated leaves were similar in both

treatments but were >50% greater in the upper non-inoculated leaves of plants inoculated with empty vector showing suppression of long distance movement of PMTV particles in *NbHIPP26* knock down plantsD, RT-qPCR quantification of RNA-Rep and RNA-TGB in upper non-inoculated leaves of plants challenged with these two PMTV genomic components (mean ± SD, n = 6, two independent experiments). *P<0.01, ** P=<0.05, Student's two tailed test.

Figure 10. Model of the mechanism of long distance movement. In a PMTV infection, TGB1 to microtubules and traffics to the nucleus where it is localises in the nucleoplasm and nucleolus (as shown in the non-vascular cell (NVC; blue). HIPP26 is mainly expressed inside the vascular bundle, predominantly in regions of the bundle containing phloem cells (phloem parenchyma, companion cells and sieve elements), and also in other vascular parenchyma cells which can sometimes be identified as xylem parenchyma (XP) due to their proximity to metaxylem elements. Once the virus reaches a vascular bundle where HIPP26 is expressed, the TGB1 binds near the CVVM (prenyl domain) of the *Nb*HIPP26 at the plasma membrane, most likely in the vicinity of plasmodesmata. This de-S-acylates the HIPP26, which releases the *Nb*HIPP26/TGB1 complex from the plasma membrane into the cytosol, from where it localises to microtubules and subsequently moves via importin-α-directed transport to the nucleus and nucleolus where HIPP26 interacts with the transcriptional activator ZFHD1. Interaction with ZFHD1 stimulates host proteins and/or stress-response factors such as NAC transcription factors, along with molecules that increase the size exclusion limit (SEL) of the plasmodesmata at the VP:companion cell (CC) interface. These plasmodesmata are seen as a key control point for entry into the sieve element (SE):CC complex and an important barrier to systemic spread for viruses. The pore-plasmodesma units (PPU) that connect CC and SE are known to have a large SEL and so the SE:CC complex has been proposed to be a single domain where even large molecules can move relatively freely into the SE. It is difficult to differentiate between what may occur in the phloem parenchyma and companion cells; protein expression and interactions may be different or the same in these cell types, indicated by the question mark in the companion cell nucleus. The key point is that we believe the virus exploits the systemic signalling response to drought in order to gain entry into the phloem. Once inside the phloem tissue, in the presence of host proteins that gate plasmodesmata, virions or RNPs can enter the SE for systemic spread.

Supplemental Fig 1. Arabidopsis HIPP family phylogeny. Phylogenetic relationships between A44 and 45 HIPP family proteins from Arabidopsis were previously identified by de Abreu-Neto (de Abreu-Neto et al., 2013). The symbols at each node denote five HIPP families (de Abreu-Neto et al., 2013). Diamond: group I, triangle: group II, white circle: group III, black circle: group IV, square: group V. The phylogeny was reconstructed using the neighbour-joining method in MEGA6 (Tamura et al., 2013) using a bootstrap mode with 1000 replications. The tree shown is the bootstrap consensus.

Supplemental Fig. 2. *Cis* regulatory elements upstream of the HIPP26 ORF. Schematic representation of potential *cis* regulatory elements involved in stress responses identified in the nucleotide sequence 0-2 kbp upstream of the predicted *NbHIPP26* ORF and identified using the PLACE database (www.dna.affrc.go.jp/PLACE/). Predicted *cis* regulatory elements are shown by coloured bars and their location with respect to the initiation codon of *NbHIPP26* shown on the grey bar. CCATBOX1 (found in the promoter of heat shock protein genes), MYB (found in the promoter of dehydration-responsive genes), ERD (Early response to drought), W-Box (found in salicylic acid responsive promoters), MYC (found in the promoter of dehydration-responsive genes), ABA responsive (found in abscisic acid and stress related promoters), GT-1 Box (found in salicylic acid responsive promoters), LTRE (low temperature responsive element).

Supplementary Table. List of primers and sequences.

Supplementary Movie. Motile vesicles tagged with GFP-HIPP26. Expression of GFP-HIPP26 revealed GFP in small motile vesicles (approx. 2 µm diameter) that move through the cytoplasm and along cytoplasmic (trans-vacuolar) strands.

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