Reticulon-like properties of a plant virus-encoded movement protein

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Key Words:	plasmodesmata, cell-to-cell transport, endoplasmic reticulum, membrane curvature, reticulon, plant virus, movement protein

Reticulon-like properties of a plant virus-encoded movement protein

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Supporting Information:

- Fig. S1.** Analysis of possible effect of BMB2 on the size and morphology of Golgi stacks.
- Fig. S2.** BiFC analysis of Derlin-1 topology in the ER membrane.
- Fig. S3.** Statistical evaluation of FRAP data.
- Fig. S4.** Prediction of BMB2 topology in the ER membrane.
- Fig. S5.** Influence of RTNLB13 transient expression on the PD SEL and virus cell-to-cell movement.
- Fig. S6.** Localization of the luminal ER marker ER-mREF in PVX-POL-GFP-infected cells.
- Fig. S7.** Sequence analysis of HGSV BMB2.
- Table S1.** Primers used in this study.
- Methods S1.** Details of cloning procedures.

SUMMARY

- Plant viruses encode movement proteins (MPs) that ensure the transport of viral genomes through plasmodesmata (PD) and use cell endomembranes, mostly the endoplasmic reticulum (ER), for delivery of viral genomes to PD and formation of PD-anchored virus replication compartments.
- Here, we demonstrate that the *Hibiscus green spot virus* BMB2 MP, an integral ER protein, induces constrictions of ER tubules, decreases the mobility of ER luminal content, and exhibits an affinity to highly curved membranes. These properties are similar to those described for reticulons, cellular proteins that induce membrane curvature to shape the ER tubules. Similar to reticulons, BMB2 adopts a W-like topology within the ER membrane.
- BMB2 targets PD and increases their size exclusion limit, and these BMB2 activities correlate with the ability to induce constrictions of ER tubules. We propose that the induction of ER constrictions contributes to the BMB2-dependent increase in PD permeability and formation of the PD-associated replication compartments therefore facilitating the virus intercellular spread.
- Furthermore, we show that the ER tubule constrictions also occur in cells expressing TGB2, one of the three MPs of *Potato virus X* (PVX), and in PVX-infected cells, suggesting that reticulon-like MPs are employed by diverse RNA viruses.

Key words: plasmodesmata, cell-to-cell transport, endoplasmic reticulum, membrane curvature, reticulon, plant virus, movement protein.

Introduction

Plant RNA viruses manipulate host cell endomembranes and host protein factors to ensure intercellular movement of the viral genomes and the formation of viral replication compartments (Heinlein, 2015a; Pitzalis & Heinlein, 2017; Hong & Ju, 2017). Virus-induced membrane structures associated with viral genome replication are often considered as ‘virus factories’ that protect the viral genomes from host antiviral mechanisms and concentrate factors required for replication (Tilsner *et al.*, 2013; Laliberté & Zheng, 2014). It is broadly accepted that plant virus replication is coupled with cell-to-cell transport through plasmodesmata (PD) and that the ‘virus factories’ can be located in the vicinity of PD (Tilsner & Oparka, 2012; Tilsner *et al.*, 2013; Heinlein, 2015a). PD are unique to plants and represent cytoplasmic channels with a diameter of about 30-50 nm that cross the plant cell walls to connect neighboring cells. The PD channels represent the main pathway for intercellular exchange of molecules (Bayer *et al.*, 2014; Otero *et al.*, 2016) and it is known since many years that they are exploited by plant viruses for their cell-to-cell movement (Tomenius *et al.*, 1987; Wolf *et al.*, 1989; Heinlein, 2015b). The PD channel has a cytoplasmic space delimited by the plasma membrane (PM) and an axial appressed tubule of endoplasmic reticulum (ER) termed ‘desmotubule’, therefore looking like a system of two membrane tubes inserted one into the other (Bell & Oparka, 2011; Liesche *et al.*, 2019). The PM and desmotubule membranes are located close to each other, at a distance of ~10-15 nm, and the cytoplasmic, inter-membrane space contains spoke-like, presumably proteinaceous, elements that emanate from the desmotubule and link the two membranes (Roberts & Oparka, 2003; Petit *et al.*, 2020). Recent studies have demonstrated that PD contain a number of proteins characteristic of membrane contact sites (MCSs) suggesting that the internal PD structure can be viewed as a specialized MCS (Tilsner *et al.*, 2016; Pérez-Sancho *et al.*, 2016; Pankratenko *et al.*, 2020; Prinz *et al.*, 2020; Ishikawa *et al.*, 2020).

To enable their cell-to-cell transport through PD, plant viruses encode movement proteins (MPs). These proteins participate in the formation of virus replication sites on cell endomembranes and are required for both the intracellular targeting of virus genomes or particles to PD and modifying the PD size exclusion limit (SEL) in order to allow the virus passage into adjacent cells (Heinlein, 2015a). For these functions, the MPs of many viruses interact with ER membranes. According to the current model of *Tobacco mosaic virus* (TMV) cell-to-cell transport, the TMV MP interacts with virus RNA, ER membranes, and microtubules to provide

sites for attachment of virus RNA-dependent RNA polymerase (replicase) and formation of virus replication complexes (VRC) at microtubule-anchored sites of the cortical ER/actin network. At early stages of infection, some of the VRCs detach from their anchoring sites and move along the cortical ER/actin network to PD vicinity aided by class XI myosin motors (Kawakami *et al.*, 2004; Amari *et al.*, 2014; Heinlein, 2015a; Pitzalis & Heinlein, 2017). Recent studies have revealed that interaction with the membrane protein SYT1, a component of MCSs, is required for TMV MP accumulation at PD during virus infection (Uchiyama *et al.*, 2014; Levy *et al.*, 2015; Yuan *et al.*, 2018).

The recruitment of virus genomes from VRCs-associated ER sites at the PD entrances for cell-to-cell movement is apparently used also by other plant viruses. In particular, Potato virus X (PVX, genus *Potexvirus*), which has three MPs encoded by a 'triple gene block' (TGB) and termed TGB1, TGB2, and TGB3 (Morozov & Solovyev, 2003) uses "co-replicative insertion" of progeny virus genomes into the PD channels from PD-anchored VRCs (Tilsner *et al.*, 2013; Wu *et al.*, 2018). These VRCs gradually recruit reorganized, densely reticulated ER membranes that serve as sites of both replication and translation of virus RNA. Therefore, the nascent progeny RNA molecules can interact with locally synthesized virus proteins and are directed to the PD channel (Tilsner *et al.*, 2013). The VRC anchoring at PD is likely mediated by TGB2 and TGB3, which are integral membrane proteins containing autonomous PD-targeting signals (Schepetilnikov *et al.*, 2005; Tilsner *et al.*, 2013). TGB3 targeting to PD depends on its oligomerization (Shemyakina *et al.*, 2011; Wu *et al.*, 2011), which is also required for the observed affinity of this protein to curved membranes manifested by TGB3 accumulation in cortical ER tubules and exclusion from flat ER cisternae (Lee *et al.*, 2010; Wu *et al.*, 2011). However, the molecular mechanisms involved in TGB3 targeting to and anchorage at PD are not known.

Thus, the dynamic ER network of membrane cisternae and tubules plays a central role in virus transport from cell to cell. The ER morphology is formed, maintained and modulated by a number of proteins known as reticulon, atlastin-like, and Lunapark (LNP) proteins (Sparkes *et al.*, 2009; Hawes *et al.*, 2015; Kriechbaumer *et al.*, 2018). Atlastin-like GTPases of the ROOT HAIR DEFECTIVE3 (RHD3) family are involved in membrane fusion and formation of three-way junctions in the polygonal network of ER tubules (Stefano & Brandizzi, 2014; Sun & Zheng, 2018). Reticulons act in the formation of ER tubules through generating membrane

curvature. This property is specified by the reticulon homology domain (RHD), a protein region comprising two pairs of transmembrane domains (TMDs) connected by a loop. This structure of the RHD domain determines a W-like topology of reticulons within the ER membrane with both protein termini and the central loop located at the cytosolic side of the membrane. The RHD TMDs of reticulons form wedge-like hairpins, which, upon reticulon oligomerization, bend the lipid bilayer (Voeltz *et al.*, 2006; Shibata *et al.*, 2008; Sparkes *et al.*, 2010; Tolley *et al.*, 2010). Membrane shaping by the reticulon RTNLB13 also depends on an amphipathic helix (Breeze *et al.*, 2016). The reticulons are believed to play the central role in inducing membrane curvature, since a purified reticulon has been shown to be sufficient to convert flat lipid bilayers into tubules (Hu *et al.*, 2008). The LNP proteins, such as *A. thaliana* AtLNP1 and AtLNP2, are involved in the formation of ER cisternae and, together with reticulons, may play important roles in maintaining the balance between ER tubules and cisternae (Kriechbaumer *et al.*, 2018).

The *A. thaliana* genome encodes 21 predicted reticulons (RTNLBs) (Nziengui *et al.*, 2007; Sparkes *et al.*, 2010), and most of these proteins remain to be studied experimentally. Two *A. thaliana* reticulons, RTNLB3 and RTNLB6, are recruited to the cell plate during cell division when primary PD are formed in the cell wall and at least RTNLB6 is associated with the PD desmotubule in mature cell walls (Knox *et al.*, 2015). Both RTNLB3 and RTNLB6 interact *in vivo* with a number of PD-associated proteins, as well as with SYT1 and VAP27 proteins, structural components of MCSs presumed to act as tethers between the desmotubule and the PM within PD (Kriechbaumer *et al.*, 2015). Therefore, the available data indicate that RTNLB3 and RTNLB6 are involved in both shaping the desmotubule in newly-formed cell walls and in maintaining the internal structure of the PD channel.

Recently, a novel transport gene module consisting of two genes and termed "binary movement block" (BMB) has been found in the genome of *Hibiscus green spot virus* (HGSV, genus *Higrevirus*, family Kitaviridae) (Melzer *et al.*, 2012). The BMB-encoded proteins BMB1 and BMB2 are distantly related to TGB1 and TGB2 proteins, respectively (Morozov & Solovyev, 2012, 2015; Solovyev & Morozov, 2017). BMB2, a small integral ER protein, localizes to PD-associated sites and induces the formation of ER-derived bodies in close vicinity of PD. Moreover, BMB2 recruits diffuse cytosolic BMB1 to these membrane bodies and to PD, and enables BMB1 spread to neighboring cells (Lazareva *et al.*, 2017b). The intracellular transport of BMB2 to PD-associated sites is independent of the ER-Golgi secretory pathway but

requires a functional ER/actin network (Lazareva *et al.*, 2017a). However, the ER/actin-associated mechanism by which BMB2 facilitates transport to and through PD is not known.

In this work, we show that BMB2 induces constrictions along ER tubules, decreases the mobility of ER luminal content, exhibits an affinity to highly curved membranes and has a W-like topology within the ER membrane, thus indicating that this protein has reticulon-like properties. Moreover, we demonstrate that the induction of ER tubule constrictions by BMB2 correlates with the ability of this protein to target PD and to increase the SEL of the PD channel. These findings lead to the proposal that HGSV and potentially other viruses target and modify curved ER membranes, particularly the desmotubule, to facilitate their movement.

Materials and Methods

Plant material

The wild type *Nicotiana benthamiana* plants and ER-GFP-transgenic *N. benthamiana* plants of line 16C were grown in a greenhouse or growth chambers under 16-h/8-h day/night periods, 24/20°C day/night temperatures and approximately 50% humidity. 5-6 weeks old plants were used for transient expression and microscopy and 6-8 weeks old plants were used for movement complementation assays. During experiments plants were kept in growth chambers.

Molecular cloning and recombinant constructs

The constructs for the transient expression of BMB1, BMB2 and its N- and C-terminal fusions to fluorescent proteins, ST-GFP, ER-GFP, ER-mRFP, PVX-POL-GFP (Lazareva *et al.*, 2017b,a), VAP27-mRFP (Atabekova *et al.*, 2018), as well as constructs YN, YC, YN-ER and YC-ER used for membrane protein topology studies (Zamyatnin Jr. *et al.*, 2006) were described previously. To obtain binary vectors carrying PVX TGB2 and TGB3 genes, the respective fragments from constructs pRT-12K and pRT-8K (Solovyev *et al.*, 2000) were subcloned into pLH* (Solovyev *et al.*, 2013). Coding sequences of RTNLB13 and Der1 were amplified on the template of *A. thaliana* total leaf RNA by RT-PCR with gene-specific primers (Table S1) and cloned into pLH* and pBlueScript SK(-), respectively. Next, Der1 was subcloned into pLH* as a C-terminal fusion with either GFP, or YC sequence. The YC-BMB2, BMB2-YC and BM<YC>B2 constructs were generated by modification of mRFP-BMB2, BMB2-mRFP and BMB2 constructs (Lazareva *et*

al., 2017b), respectively. Details of cloning procedures are described in Methods S1. All constructs were verified by sequencing.

Agroinfiltration of plants

Transformation of *Agrobacterium tumefaciens* (C58C1) with binary vectors was performed by freeze-thaw method. Agrobacterial cultures for infiltration were prepared as described previously (Solovyev *et al.*, 2013). Briefly, agrobacterial overnight cultures were grown at 28°C in LB medium supplemented with 10 mM 2-(N-morpholino)ethanesulfonic acid (MES, pH 5.5), 20 mM acetosyringone and selective antibiotics. Agrobacteria cells were centrifugated, resuspended in infiltration medium (10 mM MES, pH 5.5, 10 mM MgCl₂, 150 mM acetosyringone) and kept at room temperature for 3 to 4 hours. Prior to infiltration, agrobacterium cell suspensions were diluted to the final optical density of 0.3 at 600nm (OD₆₀₀). For co-agroinfiltration experiments, equal proportions of individual agrobacterial cultures diluted to OD₆₀₀ 0.3 were mixed together. To obtain individually transformed plant cells, high dilutions of agrobacteria were used. For virus movement complementation test, the mixed agrobacterial suspension used for plant infiltration contained the cultures carrying PVX-POL-GFP construct at OD₆₀₀ 0.00005, the cultures carrying BMB1 construct at OD₆₀₀ 0.15, and the cultures carrying BMB2 constructs (BMB2, BMB2-mRFP, or mRFP-BMB2) at OD₆₀₀ 0.15, as described earlier (Lazareva *et al.*, 2017b). To analyze alterations of the PD SEL, the culture carrying PVX-POL-GFP construct was added at OD₆₀₀ 0.0004, whereas cultures for tested constructs - at OD₆₀₀ 0.3. Final suspensions of agrobacteria were infiltrated into abaxial sides of young fully expanded leaves using a 2-ml syringe without a needle.

Confocal microscopy and FRAP analysis

Fluorescent proteins were visualized at 1 - 3 days after agroinfiltration in abaxial epidermal cells. Excised leaf sections were vacuum infiltrated with water to remove excess of air inside leaf and then observed using a Zeiss LSM780 (Oberkochen, Germany) confocal laser scanning microscope equipped with a x40 (1.4 NA) oil immersion objective. The excitation wavelengths were 488 nm for GFP, 555 nm for mRFP and 405nm for aniline blue staining. Images were also acquired on a Nikon C2plus (Japan) confocal laser scanning microscope using x60 (1.2 NA) water immersion objective and C2-DUS spectral detector. Fluorescence was excited using 403

nm for aniline blue, 488 nm for YFP and 561 nm for mRFP and detected using a band-pass dichroic mirror (405/488/561/640nm). In Z-series of optical sections, consecutive sections were taken with an interval of 2 μ m. In FRAP experiments, infiltrated leaf sections were treated with latrunculin B (20 μ M) and then imaged using a Zeiss LSM780 laser scanning confocal microscope. Five pre-bleaching frames were acquired, after that selected area of cortical ER was bleached by a combination of 405 and 488 nm lasers with 5 iterations at maximal intensity. Subsequent recovery was monitored at 0.97 sec intervals for 1 minute at 5 % 488 nm laser intensity. The microscope settings, including bleaching area, were kept constant throughout all experiments. The relative fluorescence intensity for each individual FRAP experiment was background corrected and normalized to 100% prior to bleach. The images were processed using Zeiss Zen, Nikon NIS Elements and ImageJ (1.47s) software. All experiments involving confocal microscopy were done at least 3 times. For statistical analyses of data, non-paired two-tailed Student's t-test was applied. Quantitative analysis of the overlap of mRFP and GFP signals was carried out with the JACoP plugin for ImageJ (Bolte & Cordelières, 2006) used for object-based co-localization analysis and calculation of Manders' overlap coefficient (Manders *et al.*, 1992).

Aniline blue staining

PD-associated callose was stained as described (Amari *et al.*, 2014). Leaf discs were vacuum infiltrated with aniline blue solution (0.1% aniline blue in 67 mM phosphate buffer, pH 8.0). Leaf sections were incubated in the dark at room temperature for 30 min before microscopy.

Results

BMB2 expression can alter the localization of a luminal ER marker

To investigate potential effects of BMB2 on ER membrane structure, this protein was expressed by *Agrobacterium* infiltration in epidermal cells of transgenic *Nicotiana benthamiana* plants of line 16c expressing ER-GFP (Ruiz *et al.*, 1998), a soluble marker for the ER lumen (Boevink *et al.*, 1999). Leaf epidermal cells of this line show the typical ER morphology, with the GFP fluorescence being localized to the nuclear envelope and the cortical network of ER

tubules (Fig. 1a). In the presence of BMB2, the luminal marker accumulated within the large, ER-derived peripheral bodies (Fig. 1b, c), which are typical for BMB2 expression in plant cells (Lazareva *et al.*, 2017b). Moreover, although otherwise normal ER network structure was still visible, the distribution of the fluorescent luminal marker became concentrated in local foci, conferring to the cortical ER network a “beads-on-a-string” appearance (Fig. 1b, c). Similar effects were observed when BMB2 and ER-GFP were co-expressed in wild type *N. benthamiana* plants. Control plants expressing luminal ER-GFP together with an empty control vector showed the typical distribution of the marker, thus evenly highlighting a normal ER network (Fig. 1d). However, upon co-expression of ER-GFP with BMB2, the formation of peripheral bodies and the “beads-on-a-string” appearance of the ER network were again observed (Fig. 1e).

The altered localization of luminal ER-GFP marker in BMB2-expressing cells is reminiscent of the previously reported changes in the distribution of ER-GFP induced by transient overexpression of plant reticulons (Tolley *et al.*, 2008; Sparkes *et al.*, 2010). Expression of the *Arabidopsis thaliana* reticulon RTNLB13 in ER-GFP-transgenic plants indeed led to a “beads-on-a-string” appearance of the ER network similar to expression of BMB2 (Fig. 1f). Expression of BMB2 or RTNLB13 had no visible effect on appearance of Golgi complexes (Fig. S1), as could be observed by co-expression of the Golgi marker sialyltransferase-(ST)-GFP (Lazareva *et al.*, 2017b). Collectively, these data indicate that BMB2 changes the localization of luminal ER-GFP without affecting the downstream Golgi membrane compartment, as previously reported for RTNLB13 (Tolley *et al.*, 2008).

BMB2 expression induces local constrictions in cortical ER tubules

The effect of ectopically expressed RTNLB13 on the localization of ER luminal marker has been demonstrated to result from RTNLB13-induced local ER tubule constrictions dividing the luminal content into distinct vesicle-like ER subdomains without disrupting the integrity of ER tubule membrane (Tolley *et al.*, 2008). To determine whether BMB2 could have a similar effect on ER tubules, we labeled the ER membrane by expression of *A. thaliana* Derlin1 (Der1) (Lee *et al.*, 2013), an integral ER protein with four transmembrane segments and with its N- and C-termini presumably located in the cytoplasm (Katiyar *et al.*, 2005). Expression of Der1 fused to GFP at its C-terminus (Der1-GFP) together with luminal ER-mRFP marker (Lazareva *et al.*, 2017a) led to overlapping patterns of red and green fluorescence highlighting the normal

structure of the cortical ER network (Fig. 2a-c) in accord with the previously described localization of *A. thaliana* Derlin1 to the ER membranes (Katiyar *et al.*, 2005). To verify that the C-terminus of Der1 with its fused GFP is in the cytoplasm and not in the ER lumen we used a bimolecular fluorescence complementation (BiFC) (Zamyatnin Jr. *et al.*, 2006) approach. Here, the GFP moiety of Der1-GFP was replaced with YC, a non-fluorescent C-terminal fragment of YFP (YFP₁₅₅₋₂₃₉), and expressed together with YN, the non-fluorescent N-terminal fragment of YFP (YFP₁₋₁₅₄). YN was expressed either freely and localized in the cytoplasm or as an ER-targeted version (YN-ER) located in the ER lumen (Zamyatnin Jr. *et al.*, 2006). BiFC, the reconstitution of fluorescent YFP from the two YN and YC halves, was observed upon co-expression of Der1-YC with YN but not upon co-expression of Der1-YC with YN-ER (Fig. S2). This observation shows that the Der1 C-terminus and, thus, also the fused YC or GFP moieties are oriented towards the cytoplasm, and therefore ER tubule constrictions and effects on the distribution of luminal content should not influence the localization of Der1-GFP. When BMB2 was co-expressed with both Der1-GFP and ER-mRFP, the luminal marker ER-mRFP showed the “beads-on-a-string” appearance (Fig. 2e) similar to that seen for ER-GFP co-expressed with BMB2, although the visualization of the intact ER tubule continuity required higher gain settings for analysis. Der1-GFP, in contrast, retained its uniform distribution along the ER tubules (Fig. 2d). Interestingly, object-based co-localization analysis of cell images (Bolte & Cordelières, 2006) revealed that as much as $46.3 \pm 5.2\%$ ($n=3$) ER-mRFP-containing “beads” localized to the nodes of the ER network (Fig. 2f). Taken together, these observations demonstrate that BMB2 does not affect the integrity of ER tubules but causes the displacement of luminal proteins to vesicle-like ER subdomains often located at the ER nodes.

The BMB2-induced localization of the luminal proteins to ER subdomains is likely the result of a BMB2-induced increase in membrane curvature leading to tubule constrictions that leave little space for the luminal content. To further investigate this hypothesis we used fluorescence recovery after photobleaching (FRAP) to determine whether BMB2 expression reduced the mobility of luminal content as previously demonstrated for RTNLB13 (Tolley *et al.*, 2008). Prior to FRAP, leaf fragments were treated with latrunculin B to inhibit the actin cytoskeleton and the microfilament-dependent dynamics of the ER network (Brandizzi *et al.*, 2002). As expected, RTNLB13 expression resulted in a strong reduction of fluorescence recovery compared to control (Fig. 2g) (Tolley *et al.*, 2010; Breeze *et al.*, 2016). Importantly, a

similar, although less pronounced, effect was observed upon BMB2 expression (Fig. 2g). The observed differences between the control and both BMB2 and RTNLB13 were statistically significant, whereas the difference between BMB2 and RTNLB13 was insignificant (Fig. S3). This experimental outcome indicates that BMB2 restricts the flow of luminal content similar to RTNLB13, supporting the hypothesis that BMB2 expression induces the constriction of ER tubules.

mRFP-BMB2 fusion protein localizes to the sites of ER tubule constrictions

Next, we employed BMB2 protein fused to mRFP to determine the spatial relationship between BMB2 and the BMB2-induced ER constrictions. mRFP-BMB2 was found to localize to numerous punctate structures in the cytoplasm in addition to the cell wall-associated bodies (Fig. 3a-f). Closer inspection of mRFP-BMB2-expressing cells revealed the presence of ER constrictions, as indicated by the concentration of the ER-GFP marker at network nodes, as well as uneven or discontinuous ER tubules (Fig. 3d-f). Moreover, the punctate mRFP-BMB2 structures were associated with the ER structures (Fig. 3f), being mostly located to ER tubule locations depleted for ER-GFP, presumably because of the induction of local ER tubule constrictions that fully displaced ER-GFP from these sites (Fig. 3d-f). Consistently, by merging the mRFP-BMB2 and ER-GFP images it can be seen that mRFP-BMB2 exactly localizes to these ER sites of local luminal ER-GFP displacement (Fig. 3d-f). These observations were confirmed by image quantification, showing that the specific location of mRFP-BMB2 had little spatial overlap with the neighboring ER-GFP-containing regions in the ER tubules, as Manders' overlap coefficient showing the proportion of mRFP signal overlapping ER-GFP signal in these cells was calculated to be 0.23 ± 0.04 ($n=3$). The finding that mRFP-BMB2 accumulates to discrete sites of cortical ER depleted of luminal ER-GFP supports the hypothesis that BMB2 induces local ER tubule constrictions and suggests that constrictions are associated with BMB2 localization at the constriction sites.

Interestingly, BMB2-mRFP, another fusion protein with mRFP fused to the C-terminus of BMB2, was detected in ER-GFP-containing peripheral bodies, as described previously (Lazareva *et al.*, 2017b); however, no association of BMB2-mRFP with tubules of the cortical ER was detected, and the localization of ER-GFP was not affected, showing no visible signs of constrictions (Fig. 3g-i). This observation suggests that the ability of BMB2 to induce ER tubule

constrictions can be uncoupled from formation of peripheral bodies and inhibited by fusion of mRFP to the BMB2 C-terminus. To confirm the finding that the two fusion proteins differ in their abilities to induce ER tubule constrictions, we used FRAP analysis. The degree of fluorescence recovery in the bleached ER regions was reduced in cells expressing BMB2 and mRFP-BMB2 as compared to the control cells, whereas in cells expressing BMB2-mRFP the pattern of fluorescence recovery was closer to the control (Fig. 3j). The differences of both BMB2 and mRFP-BMB2 from the control, but not that of BMB2-mRFP, were statistically significant, and the difference between the two fusion proteins was also significant (Fig. S3). Therefore, these data support the conclusion based on the microscopical observations that mRFP-BMB2, but not BMB2-mRFP, is able to induce constrictions of ER tubules.

BMB2 is localized to rims of ER cisternae

Reticulons are known to localize to both ER tubules and rims of ER cisternae (Sparkes *et al.*, 2010). To determine whether also BMB2 localizes to the rim of ER cisternae in addition to ER tubules, we changed ER structure by expression of NbVAP27-mRFP (Atabekova *et al.*, 2018). This protein localizes to the ER network (Fig. 4a-c); however, in some cells its expression causes the reorganization of the cortical ER network into a system of interconnected membrane cisternae (Fig. 4d-f). GFP-BMB2 associated with ER tubules and induced constrictions similar to mRFP-BMB2 (Fig. 4g-i). When analyzed in the presence of NbVAP27-mRFP, GFP-BMB2 localized to the rims of the NbVAP27-induced cisternae. GFP-BMB2 had a clear preference for the rims with high membrane curvature as almost no signal could be detected on the flat surface of the membrane sheets (Fig. 4j-l). These observations show that BMB2 has an affinity to highly curved ER membranes and induces further membrane curvature resulting in ER tubule constrictions, thus showing properties typical for reticulons (Sparkes *et al.*, 2010).

BMB2 topology in the ER membrane

Next, we wanted to determine whether BMB2 also shares structural features with reticulons. Reticulons induce membrane curvature by oligomerization and by hairpin structures of their conserved RHD region forming a double wedge, W-like, topology in the membrane (Voeltz *et al.*, 2006; Hu *et al.*, 2008; Shibata *et al.*, 2008). BMB2 forms high-molecular-mass complexes *in vivo* (Lazareva *et al.*, 2017b) and has been predicted by sequence analysis to

possess two TMDs (Morozov & Solovyev, 2012) (Fig. 5a). Topology prediction with a hidden Markov model (Krogh *et al.*, 2001) suggested that BMB2 could have a U-like topology in the ER membrane, with the N- and C-termini of the protein being located in the cytoplasm, and its central hydrophilic region in the ER lumen (Fig. S4). To experimentally assess the BMB2 topology, a BiFC assay was used in which YC was fused to either the N- or C-terminus of BMB2 (YC-BMB2 and BMB2-YC, respectively), or inserted in the BMB2 central hydrophilic region (BM<YC>B2) (Fig. 5a). Each of the three fusions was individually co-expressed with either YN, or YN-ER. YFP fluorescence was observed upon co-expression of YC-BMB2, BMB2-YC or BM<YC>B2 with YN, whereas no YFP fluorescence was observed when any of these BMB2 constructs was co-expressed with YN-ER (Fig. 5b). In controls, YN produced fluorescence when co-expressed with YC but not YC-ER, and YN-ER produced YFP fluorescence when co-expressed with YC-ER but not with YC (Fig. 5b), thus demonstrating expression and functionality of all BiFC reporter constructs (YN, YC, YN-ER, YC-ER). The YFP signals produced upon co-expression of the BMB2 constructs with YN localized to bodies at the cell periphery (Fig. 5b) reflecting the accumulation pattern observed for BMB2 fusions to mRFP (Fig. 3b, 3h). Taken together, these BiFC data show that the N- and C-termini of BMB2, as well as the central hydrophilic region of the protein, are located in the cytoplasm and suggest that the protein has a W-like, rather than U-like, topology in the ER membrane (Fig. 5c).

ER constrictions induced by BMB2 correlate with PD targeting and SEL increase

To determine whether the ability of BMB2 to induce ER tubule constrictions is functionally linked to other functions of this MP, we compared the functional properties of mRFP-BMB2 capable of inducing constrictions (Fig. 3a-f, j) with those of BMB2-mRFP, which neither localizes to ER tubules, nor induces any constrictions (Fig. 3g-j) and can be considered as a negative control. mRFP-BMB2 was found to localize to both peripheral bodies and structures at or near PD identified by callose staining (Fig. 6a), whereas BMB2-mRFP localized to cell wall-appressed bodies but not to the callose depositions in the cell wall (Fig. 6b). These observations correlate the ability of mRFP-BMB2 to induce ER tubule constrictions with its localization to callose depositions in the cell wall, thus suggesting its ability to target PD, whereas BMB2-mRFP, which is not able to induce ER tubule constrictions, is incapable of PD targeting.

To determine which of the HGSV MPs could have the ability to increase the PD size exclusion limit (SEL), they were individually tested in a GFP diffusion assay. BMB1 or BMB2 was transiently expressed in leaves in which single cells were agroinfected with PVX-POL-GFP amplicon producing high levels of cytoplasmic GFP but incapable of cell-to-cell transport due to deletion of TGB and CP genes (Lazareva *et al.*, 2017b). As both BMB1 and BMB2 are required for virus movement, the individual expression of the BMB proteins does not result in PVX-POL-GFP cell-to-cell movement (Lazareva *et al.*, 2017b), and therefore can be used to determine PD permeability exhibited by the rate of diffusion of GFP from the PVX-POL-GFP-infected cells to adjacent cells. At 3 dpi, the GFP diffusion out of the individual GFP-expressing cells was estimated as a percentage of fluorescent loci, in which the GFP fluorescence was observed in two or more cells. In the presence of empty expression vector, the percentage of such loci was 26.8, whereas expression of BMB1 and BMB2 resulted in 16.5% and 65.1% such loci, respectively (Fig. 6c-e, h). Therefore, BMB2, but not BMB1, is able to increase the PD SEL and facilitate GFP diffusion out of infected cells. Upon expression of BMB2-mRFP, GFP diffusion was similar to that seen with empty vector, with the difference between vector and BMB2-mRFP being statistically insignificant according to Student's t-test ($P = 0.68$). On the other hand, expression of mRFP-BMB2 led to 68.3% loci with GFP diffusion, similar to the GFP diffusion induced by unfused BMB2 (Fig. 6f-h), showing a significant increase compared to empty vector ($P < 0.001$ for both BMB2 and mRFP-BMB2). These observations correlate the ability of mRFP-BMB2 to induce ER tubule constrictions and to localize to PD with its ability to increase the PD SEL. In contrast, BMB2-mRFP, which fails to localize to ER tubules, to induce ER constrictions and to localize to PD, also fails to increase the PD SEL. These correlations suggest a functional link between the BMB2-induced ER constrictions and either PD targeting, PD modification, or both. It should be noted that transient expression of RTNLB13 was found to have no influence on the PD SEL or virus cell-to-cell movement (Fig. S5), suggesting that the ability to induce constrictions, on its own, is not sufficient for the PD targeting and/or PD modification, which therefore may involve other, BMB2-specific functions.

PVX TGB2 is also capable of inducing ER tubule constrictions

The BMB2 protein is distantly related to the hydrophobic movement protein TGB2 encoded in genomes of a number of plant viruses (Morozov & Solovyev, 2012, 2015; Solovyev

& Morozov, 2017) suggesting that these proteins may share ability to induce constriction of ER tubules. To test this possibility, we expressed the PVX TGB2 in transgenic *N. benthamiana* plants producing the luminal ER-GFP marker. Indeed, the cortical ER network appeared disintegrated into numerous ER-GFP-containing vesicular structures (Fig. 7a). At increased gain settings and elevated image brightness, faint ER tubules connecting these structures became visible (Fig. 7b). Therefore, ER-GFP localization in these cells showed a “beads-on-a-string” pattern similar to the pattern induced by BMB2. In contrast, expression of the PVX TGB3 protein induced the formation of peripheral ER-derived bodies as described previously (Schepetilnikov *et al.*, 2005), but did not affect the normal localization of ER-GFP in the lumen of ER tubules (Fig. 7c). These observations suggest that the PVX TGB2 protein, but not the TGB3 protein, may cause constrictions of ER tubules similarly to BMB2.

To determine whether the ER tubule constrictions also occur during PVX infection, the localization of luminal ER marker ER-mRFP was analyzed in cells infected with GFP-tagged PVX (PVX-GFP) (Santa Cruz *et al.*, 1996). Observation of infected leaf tissues at 2 dpi revealed that ER-mRFP was localized in numerous small structures scattered over the cytoplasm (Fig. 7d-f). At higher image brightness, these bodies appeared interconnected by ER tubules and localized predominantly at the nodes of the ER network (Fig. 7g). Therefore, PVX infection involves the constriction of ER tubules. As the “beads-on-a-string” appearance of ER tubules was not observed in PVX-POL-GFP-infected cells (Fig. S6), we conclude that the constrictions are induced by PVX non-replicative proteins, likely by TGB2, rather than by virus replication or an infection-related stress.

Discussion

The data presented in this paper show that BMB2, the integral membrane MP encoded by HGSV, has properties typical of reticulons, namely an ability to target highly curved membrane regions and to induce constrictions of ER tubules thus causing the redistribution of luminal contents. Previously, BMB2 was shown to form high-molecular-mass complexes in plant cells (Lazareva *et al.*, 2017b) that is consistent with oligomerization, another known property of reticulons (Shibata *et al.*, 2008). As we also demonstrate, the ER tubule constriction activity of HGSV BMB2 is shared by the distantly related PVX TGB2, that is in agreement with earlier observations showing that PVX TGB2 is localized to the ER tubules and ER-derived vesicular

structures associated with ER tubules (Mitra *et al.*, 2003; Ju *et al.*, 2005). Collectively, these data imply that ER constriction could be a general mechanistic aspect of plant virus cell-to-cell movement.

Constrictions of ER tubules upon high-level expression of reticulons resulted from the ability of reticulons to generate membrane curvature (Tolley *et al.*, 2008; Sparkes *et al.*, 2010). This property was shown to depend mainly on the W-like topology of the reticulon RHD region, which comprises two wedge-like hydrophobic hairpins integrated into the ER membrane, as well as on the ability of reticulons to oligomerize (Hu *et al.*, 2008; Shibata *et al.*, 2008; Tolley *et al.*, 2010). Our results are consistent with the hypothesis that BMB2 may use a similar mechanism to induce membrane curvature. The BMB2 protein has two predicted transmembrane regions, and our BiFC analysis of BMB topology in the ER membrane indicates that the protein has a W-shaped conformation, with both its termini and the central region located in the cytoplasm, as known for reticulons (Voeltz *et al.*, 2006). Based on available data we suggest that PVX TGB2 could have a similar topology. Consistently, both PVX TGB2 termini have been localized to the cytoplasm (Lee *et al.*, 2010). Although not addressed experimentally, the central hydrophilic domain may also be localized to the cytoplasm, as both this region and the C-terminal domain are shown to interact with the viral replicase (Wu *et al.*, 2018). The BSMV TGB2 may also have a BMB2-like W-shaped topology, as the central region of this protein has been shown to interact with the central hydrophilic region of BSMV TGB3 supposed to be located in the cytoplasm (Lim *et al.*, 2008).

A W-like topology implies that both BMB2 hydrophobic regions are submerged into the ER membrane, and each of them makes a turn within the lipid bilayer, thus forming an intramembrane ‘re-entrant loop’ (Yan & Luo, 2010) that does not fully traverse the lipid bilayer. Currently, there are no methods for reliable prediction of re-entrant loops and helices in membrane proteins (Leman *et al.*, 2015), and this type of interaction with the ER membrane can only be identified experimentally, as has been described for a number of proteins, such as atlastins (Betancourt-Solis *et al.*, 2018), human RTN4 and caveolin-1 (Zurek *et al.*, 2011). Importantly, the two hydrophobic BMB2 regions (predicted to be 16 and 22 residues in length, Fig. S4, Fig. S7) are considerably shorter than the RHD regions forming the reticulon-specific hydrophobic hairpins (28-36 residues in length) (Di Sano *et al.*, 2012), therefore the interaction of BMB2 with the ER membrane may differ from that of reticulons. We hypothesize that the N-

terminal BMB2 hydrophobic fragment, which is delimited by two proline residues and predicted to be of 16 residues in length (Fig. S7), may be too short to form a transmembrane α -helix, as the TMD length in ER-resident proteins is known to be 22.8 ± 6.7 residues (Singh & Mittal, 2016). Thus, interaction of this hydrophobic region with the ER membrane may result in formation of re-entrant loop, which is predicted to lack, fully or partially, an α -helical conformation (Fig. S7). On the other hand, the C-terminal BMB2 hydrophobic fragment confidently predicted as a helix may form kinks due to internal glycine residues (Fig. S7), as glycines are known to confer a high degree of local flexibility on polypeptides and are frequently found in protein regions where the polypeptide chain makes a sharp turn (Franklin & Slusky, 2018). It should be also noted that neither hydrophilic, nor membrane-embedded regions of BMB2 or TGB2 have a sequence similarity to reticulons, suggesting a reticulon-independent evolutionary origin of viral proteins with reticulon-like properties.

Diverse positive-strand RNA viruses that replicate in the cytoplasm in association with modified endomembranes recruit reticulons for membrane remodeling and formation of virus replication compartments, as documented for flaviviruses and enteroviruses (Tang *et al.*, 2007; Aktepe *et al.*, 2017), as well as for *Brome mosaic virus* (Diaz *et al.*, 2010; Diaz & Ahlquist, 2012). *Vaccinia virus* even encodes its own reticulon in its DNA genome. This reticulon contains a typical RHD domain and is involved in the formation of virus replication compartments (Erlandson *et al.*, 2016). Consistently, the ER tubule constrictions induced by BMB2 and TGB2 may have a role in the formation of virus replication compartments, which in the case of PVX were shown to be composed of a condensed ER network (Tilsner *et al.*, 2013). TGB2 was shown to interact with the PVX replicase (Wu *et al.*, 2018) that may facilitate this process. However, the ability to induce the constriction of ER tubules may also play a role in virus movement given that this activity of BMB2 correlates with its ability to target PD and to increase the PD SEL. While it is conceivable that induction of ER constrictions plays a role in a yet unknown mechanism that targets the protein and HGSV genomic RNA to PD, it is also possible that this BMB2 function operates at PD. The BMB2 preference for curved membranes may facilitate its binding to the desmotubule, which is one of the most strongly curved membrane structures known in nature (Tilsner *et al.*, 2011). In binding along the desmotubule, BMB2 may cause a displacement of MCS components, disruption of MCSs and expansion of the cytoplasmic space within PD to increase the PD SEL. Such a theoretical mechanism is consistent with observations that PVX

TGB2 increases PD SEL (Tamai & Meshi, 2001) and accumulates in the central cavities inside PD (Tilsner *et al.*, 2013).

The results presented in this paper are consistent with a model (Fig. 8) in which the BMB2 protein is co-translationally integrated into the ER membrane and uses the ER-actin network (Lazareva *et al.*, 2017a) to target PD and to enter the PD channel as an integrated component of the desmotubule. Inside PD, BMB2 molecules form complexes, which may displace native PD components and therefore increase the PD SEL. Further production of BMB2 in virus-infected cells may lead to BMB2 accumulation at the PD entrance leading to the formation of membrane bodies similar to those formed by PVX TGB2 at the PD orifice (Tilsner *et al.*, 2013) and likely the targeting sites of virus replicase (Fig. 8). Thus, BMB2 may have a dual function in modifying the PD channel and in organizing PD-associated membrane compartments for virus replication, thus coupling virus replication to the cell-to-cell movement. Given the similarity in activities of the TGB and BMB-encoded proteins, it seems plausible that this model may apply to a broader range of plant viruses.

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Author contributions

A.G.S. conceived the research; E.A.L., V.V.D., S.Y.M., M.H., and A.G.S. designed the experiments; E.A.L., A.A.L., D.A.C., S.A.G., performed the research; E.A.L., V.V.D., S.Y.M., M.H., and A.G.S. wrote the article.

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Legends to figures

Fig. 1 Localization of the luminal ER marker ER-GFP is changed in the presence of BMB2. (a) Localization of ER-GFP in a leaf epidermal cell of transgenic *N. benthamiana* plant (line 16c). An arrow points to the nuclear envelope. (b, c) Localization of ER-GFP in cells of line 16c plants in the presence of BMB2 showing the characteristic "beads-on a-string" pattern. Arrowheads in (b) and (c) point to peripheral ER-derived bodies induced by BMB2-expression. The white square in (b) denotes image area shown in (c). Arrows in (c) point to local foci of ER-GFP localization. (d) Localization of ER-GFP transiently expressed in *N. benthamiana* leaf epidermal cell by agroinfiltration. (e) Localization of ER-GFP co-expressed with BMB2 by agroinfiltration. (f) Localization of ER-GFP co-expressed with RTLNB13 by agroinfiltration. All images are reconstructed from Z-series of optical sections. Bar: 10 μ m (a, b, d-f), 5 μ m (c).

Fig. 2 BMB2 expression can induce constrictions of cortical ER tubules. (a-c) Co-expression of ER-mRFP and Der1-GFP in *N. benthamiana* leaf epidermal cell. (a) Der1-GFP. (b) ER-mRFP. (c) Overlap of images in (a) in (b). (d-f) Co-expression of ER-mRFP, Der1-GFP and BMB2. (d) Der1-GFP. (e) ER-mRFP. (f) Overlap of images in D in E. (g) FRAP analysis of cortical ER in epidermal cells of leaves of ER-GFP-transgenic plants agroinfiltrated for expression of BMB2, RTLNB13, or an empty vector (control). Fluorescence intensities normalized to pre-bleaching values are plotted against time. Error bars indicate the standard error. Six independent replicates per construct were analyzed. Images in (a) to (f) are reconstructed from Z-series of optical sections. Bar: 10 μ m.

Fig. 3 mRFP-BMB2 fusion protein localizes to the sites of ER tubule constrictions. (a-f) Expression of mRFP-BMB2 in ER-GFP-transgenic *N. benthamiana* plants. (a, d) ER-GFP. (b, e) mRFP-BMB2. (c) Overlap of images in (a) and (b). (f) Overlap of images in (d) and (e). The inset in (d-f) shows an enlarged image area. (g-i) Expression of BMB2-mRFP in ER-GFP-transgenic *N. benthamiana* plants. (g) ER-GFP. (h) BMB2-mRFP. (i) Overlap of images in (g) and (h). Bar: 10 μ m (c, i), 5 μ m (f). (j) FRAP analysis of cortical ER in epidermal cells of leaves of ER-GFP-transgenic plants agroinfiltrated for expression of BMB2, BMB2-mRFP, mRFP-BMB2, or an empty vector (control). Fluorescence intensities normalized to pre-bleaching values

are plotted against time. Error bars indicate the standard error. Six independent replicates per construct were analyzed.

Fig. 4 BMB2 has an affinity to high-curvature ER membranes. (a-f) NbVAP27-mRFP expression in ER-GFP-transgenic *N. benthamiana* plants. (a-c) Cell with unaltered cortical ER structure. (d-f) Cell with induced formation of ER-derived cisternae. (a, d) ER-GFP. (b, e) NbVAP27-mRFP. (c) Overlap of images in (a) and (b). (f) Overlap of images in (d) and (e). (g-i) Co-expression of GFP-BMB2 and ER-mRFP. (g) GFP-BMB2. (h) ER-mRFP. (i) Overlap of images in (g) and (h). The inset in (g-i) shows an enlarged image area. (j-l) Co-expression of GFP-BMB2 and NbVAP27-mRFP in a cell with NbVAP27-mRFP-induced cisternae. (j) GFP-BMB2. (k) NbVAP27-mRFP. (l) Overlap of images in (j) and (k). Bar: 10 μ m (c, f, i), 5 μ m (l).

Fig. 5 Topology of BMB2 in the ER membrane. (a) Fusion proteins used in BiFC experiments. The BMB2 and YC proteins are shown as green and yellow boxes, respectively. Positions of BMB2 transmembrane domains (TMDs) are shown by hatching. (b) BiFC analysis of BMB2 topology. YC and its fusions, as indicated above images, were co-expressed with either YN-ER or YN, as shown on the left. Images in the low row represent images in the middle row superimposed on bright-field images to show the localization of BMB2-specific bodies at the cell walls. Bar: 20 μ m. (c) A model of BMB2 topology in the ER membrane based on the BiFC data.

Fig. 6 BMB2 is able to target PD and increase the PD SEL. (a-b) Co-localization of BMB2-mRFP and mRFP-BMB2 with callose. The fusion proteins were expressed in *N. benthamiana* leaves. At 1.5 dpi, leaf specimens were stained for PD-associated callose with aniline blue and subjected to microscopy. (a) mRFP-BMB2 is present in peripheral bodies and structures co-localized with callose. (b) BMB2-mRFP is present in peripheral bodies but is not co-localized with callose. (c-e) Analysis of the ability of BMB1 and BMB2 to increase the PD SEL in a GFP diffusion test. PVX-POL-GFP was co-expressed with an empty vector control (c), BMB1 (d) and BMB2 (e). (f-g) Analysis of the ability of mRFP-BMB2 and BMB2-mRFP to increase the PD SEL. PVX-POL-GFP was co-expressed with mRFP-BMB2 (f) and BMB2-mRFP (g). Bar: 5 μ m (a, b), 50 μ m (c-g). (h) Percentage of fluorescent foci consisting of two and more cells in the GFP diffusion test. Proteins co-expressed with PVX-POL-GFP are indicated above the graph.

Number of observed foci (n) was as follows: vector, 526; BMB1, 166; BMB2, 248; mRFP-BMB2, 264; BMB2-mRFP, 247.

Fig. 7 Localization of the luminal ER marker ER-GFP in cells expressing PVX TGB2 and TGB3 and in PVX-infected cells. (a, b) Localization of ER-GFP in cells expressing PVX TGB2 showing the beads-on-a-string" phenotype. The white square in (a) denotes image area shown in (b) with increased brightness. (c) Localization of ER-GFP in cell expressing PVX TGB3. Arrowheads point to peripheral ER-derived bodies characteristic of TGB3 expression. (d-f) Localization of ER-mRFP in cell infected with PVX-GFP imaged at 2 dpi. (d) Localization of ER-mRFP. The white square in (d) denotes image area shown in (g) with increased brightness. (e) Localization of GFP confirming that the shown cells are infected with PVX-GFP. (f) Overlap of images in (d) and (e). Bar: 20 μ m (c, f), 10 μ m (a, g), 5 μ m (b).

Fig. 8 Model of BMB2 functional interaction with PD. (a) BMB2 is transported to PD along the ER structures. (b) BMB2 accumulates in the desmotubule that results in displacement of PD-specific MCS components and increasing the PD SEL. As all possible positions in the desmotubule are occupied by BMB2, further transport of BMB2 into the PD channel becomes restricted (shown by a red cross). (c) Transport of additional BMB2 molecules to PD results in accumulation BMB2 at the PD entrance and formation of ER-derived bodies. (d) As the viral replicase is incorporated in BMB2-induced bodies, these structures serve as virus replication sites.

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131x87mm (300 x 300 DPI)

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132x103mm (300 x 300 DPI)

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131x140mm (300 x 300 DPI)

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171x55mm (300 x 300 DPI)