Subcellular localization and membrane association of the replicase protein of grapevine rupestris stem pitting-associated virus, family Betalflexiviridae

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As a member of the newly established Betalflexiviridae family, grapevine rupestris stem pitting-associated virus (GRSPaV) has an RNA genome containing five ORFs. ORF1 encodes a putative replicase polyprotein typical of the alphavirus superfamily of positive-strand ssRNA viruses. Several viruses of this superfamily have been demonstrated to replicate in structures designated viral replication complexes associated with intracellular membranes. However, structure and cellular localization of the replicase complex have not been studied for members of Betalflexiviridae, a family of mostly woody plant viruses. As a first step towards the elucidation of the replication complex of GRSPaV, we investigated the subcellular localization of full-length and truncated versions of its replicase polyprotein via fluorescent tagging, followed by fluorescence microscopy. We found that the replicase polyprotein formed distinctive punctate bodies in both Nicotiana benthamiana leaf cells and tobacco protoplasts. We further mapped a region of 76 amino acids in the methyl-transferase domain responsible for the formation of these punctate structures. The punctate structures are distributed in close proximity to the endoplasmic reticulum network. Membrane flotation and biochemical analyses demonstrate that the N-terminal region responsible for punctate structure formation associated with cellular membrane is likely through an amphipathic α helix serving as an in-plane anchor. The identity of this membrane is yet to be determined. This is, to our knowledge, the first report on the localization and membrane association of the replicase proteins of a member of the family Betalflexiviridae.

INTRODUCTION

Grapevine rupestris stem pitting-associated virus (GRSPaV) was discovered in 1998 (Meng et al., 1998; Zhang et al., 1998). GRSPaV is widely distributed among commercial grape varieties and rootstocks and is associated with several diseases, namely rupestris stem pitting, vein necrosis and Syrah decline (Martelli, 1993; Meng & Gonsalves, 2007). GRSPaV comprises a family of genetic variants (Meng et al., 1999, 2006; Nolasco et al., 2006; Terlizzi et al., 2010, 2011). To date, the genomes of 11 GRSPaV isolates have been sequenced (Meng et al., 1998, 2005; Zhang et al., 1998; Lima et al., 2006, 2009; Morelli et al., 2011). GRSPaV belongs to the genus Foveavirus (family Betalflexiviridae), and has a positive-strand ssRNA genome of 8725 nt encapsidated in a 723 nm-long filamentous capsid. The GRSPaV genome contains five ORFs, encoding the replicase polypeptide, three movement proteins encoded by the triple gene block (TGB), and the capsid protein (Fig. 1; Meng & Gonsalves, 2007). The deduced amino acid sequence of ORF1 contains signature domains indicative of a typical replicase protein of the Alphavirus-like superfamily of RNA viruses (Koonin et al., 1993; van der Heijden & Bol, 2002). These include a methyl-transferase (MTR), an RNA helicase and an RNA-dependent RNA polymerase domain. The replicase polypeptide of GRSPaV is unique in that, besides the widely conserved domains described above, it also includes two cysteine protease domains: the papain-like protease and the ovarian tumour protease. Moreover, it also contains an AlkB domain recently identified through bioinformatics (Bratlie & Drabløs, 2005). It is intriguing that similar AlkB domains are present only in a small number of plant RNA viruses, most of which infect woody perennials (Martelli et al., 2007). The biological function of these unique domains in these viruses remains unknown.

GRSPaV is a member of the newly established family Betalflexiviridae, which contains mostly viruses infecting woody plants, including many economically important fruit crops (Martelli et al., 2007; King et al., 2012). Due to difficulties working with woody plants and unavailability of...
effective experimental systems, understanding of this family of viruses is scarce. Evidently, a better understanding of mechanisms for GRSPaV replication will have far-reaching implications, both scientifically and economically. One of our long-term goals is to establish GRSPaV as a model system for studying viruses of the Betaflexiviridae. Determination of the intracellular location of GRSPaV proteins and their interaction with host cell factors/structures would be a first step towards this goal. Using fluorescent protein tagging and microscopy, we have investigated and revealed the subcellular locations of four proteins encoded by GRSPaV: the three movement proteins (TGBp1-3) (Rebelo et al., 2008) and the capsid protein (Meng & Li, 2010). The subcellular localization patterns of GRSPaV TGB proteins resemble their counterparts in potato virus X (PVX, genus Potexvirus; family Alphaflexiviridae), in line with their highly similar molecular structure and motif conservation (Verchot-Lubicz et al., 2010). More recently, we have constructed a full-length cDNA clone of GRSPaV and a GFP-tagged variant and shown that both viral clones were infectious in grapevine and several herbaceous experimental hosts (Meng et al., 2013). These studies set the foundation for further in-depth investigations of various aspects of GRSPaV replication and infection.

In this study, we investigated the subcellular localization and membrane association of the replicase polyprotein as a...
first step towards the characterization of the replication machinery of GRSPaV in plant cells. We show that the replicase polyprotein, and specifically its MTR domain, forms punctate structures. These punctate structures were distributed in close proximity to the endoplasmic reticulum (ER). Membrane flotation and biochemical analyses confirm the association of these structures with cell membrane. To our knowledge, this is the first report on the subcellular localization and membrane association of the replicase protein of a member of the family Betaflexiviridae.

RESULTS

Full-length replicase polyprotein forms distinctive punctate structures

To examine the subcellular distribution of GRSPaV replicase polyprotein, we expressed full-length replicase polyprotein tagged with GFP, REP-GFP, in Nicotiana benthamiana leaves via agro-infiltration, followed by confocal laser scanning microscopy (CLSM) at different time points. Representative images of infiltrated leaves showing green fluorescence due to REP-GFP are shown in Fig. 2. Green fluorescence was clearly visible 1 day post-infiltration (p.i.) and continued to increase in intensity with time until 9 days p.i. Interestingly, at early time points, the green fluorescence appeared to be more diffused in epidermal cells and was distributed mainly along the cell periphery (Fig. 2a). Starting from 2 days p.i., punctate structures, 1–4 μm in diameter, along with tubular structures, formed. The number of these structures increased with time and reached a plateau at 9 days p.i. (Fig. 2b–f). Interestingly, for many of these punctate structures, green fluorescence was seen at the contour while the centre remained void of green fluorescence (Fig. 2b–e, and close-up in D’ and G’, indicated by arrows in (b), (d), (g) and (e)). Based on the shape and size, these punctate structures looked like chloroplasts. However, they were not chloroplasts, as demonstrated by lack of co-localization with red fluorescence emitted by chlorophyll (Fig. 2g–i).

The N-terminal region of the replicase polyprotein contains sequence determinants for the formation of punctate structures

Recent research on two members of the Alphavirus-like superfamily, Semliki Forest virus (SFV, genus Alphavirus; family Togaviridae) and brome mosaic virus (BMV, genus Bromovirus, family Bromoviridae), has revealed that the MTR domain contains sequences responsible for punctate structure formation (den Boon et al., 2001; Spuul et al., 2007; Liu et al., 2009). Because GRSPaV also belongs to the Alphavirus-like superfamily, we predicted that the MTR domain of its replicase polyprotein might also contain sequences capable of forming punctate bodies in plant cells. To test this, we constructed pREP1–749-GFP, which would express the N-terminal region of the GRSPaV replicase polyprotein (aa 1–749) containing the MTR and the highly variable region (Meng & Gonsalves, 2007) (Fig. 1). Punctate structures, about 1 μm in diameter, were observed in the cytoplasm of transfected BY-2 protoplasts. Interestingly, many of these punctate bodies were located in the perinuclear region of the protoplasts (Fig. 3f, indicated by arrows), with some being present near the plasma membrane. These structures appeared similar in size and shape to those observed in protoplasts transfected with pREP-GFP [Fig. 3, compare (d) to (f)]. Similar structures were never observed in protoplasts transfected with the control, pRTL2-GFP (Fig. 3b). These data suggest

![Fig. 2](http://vir.sgmjournals.org/923)
that this N-terminal region of the replicase polyprotein of GRSPaV contains sequences responsible for the formation of punctate structures.

Bioinformatic analysis predicts a putative amphipathic α-helix sequence within MTR for in-plane membrane (IPM) association and punctate structure formation

Viral replication complexes (VRCs) of positive-strand RNA viruses generally associate with intracellular membranes, and this membrane association can be achieved in several ways. Analysis of the deduced amino acid sequence of GRSPaV ORF1 failed to identify a transmembrane domain. Hence, association of GRSPaV replicase polyprotein with an intracellular membrane would have to either involve monotopic membrane association through an amphipathic α-helix as an in-plane anchor (Sapay et al., 2006) or be mediated through a yet-unidentified integral membrane protein provided by the cell. The former scenario would be reminiscent of the membrane association of the replicase protein of SFV (Spuul et al., 2007) and BMV (Liu et al., 2009). It was shown that amino acid residues 392–409 in the SFV MTR domain are responsible for forming punctate bodies, and for associating with the ER, endosomes and lysosomes in HeLa cells (Spuul et al., 2007). In contrast, a region (residues 245–264) in the MTR domain of BMV, a plant-infecting virus of the Alphavirus-like superfamily, determines punctate body formation but not membrane association (den Boon et al., 2001; Liu et al., 2009). To test if the replicase polyprotein of GRSPaV contains a functionally similar amphipathic α-helix sequence involved in forming punctate structures and in membrane association, we first performed hydropathy analyses of the MTR domain using the Protean program (DNASTAR), which revealed several candidate regions. The region encompassing amino acid residues 165–187 (PRVISTGARNLFLHDEIHYWSIS) seemed to be the most promising for further analyses, in part because it had a sufficient length to allow formation of an amphipathic α-helix as hypothesized to be an IPM anchor for monotopic membrane association in SFV (Spuul et al., 2007). Importantly, key amino acid residues that are consistently identified in IPM anchors, namely R, F and W (Sapay et al., 2006), are also present in this region. To corroborate this initial prediction, we performed sequence analysis using HELIQUEST for predicting putative sequences that may form amphipathic α-helix structures. As a result, only one region of 41 residues (aa 147–187) was identified, which has a hydrophobic (i.e. membrane-association) face (i.e. ≥ 5 aa consecutive hydrophobic residues), and a charge of +5. The discriminating factor (D) HELIQUEST uses in predicting amphipathic α-helices depends strongly on the charge of a peptide. Based on the model by Gautier et al. (2008), \( D \geq 1.34 \) indicates presence of a reliable lipid-binding amphipathic α-helix. The D value for our sequence is 1.86, much higher than the set threshold, suggesting it robustly to be a membrane-binding, amphipathic α-helix (Fig. 4). Therefore, we hypothesized that the region aa 147–187 would be responsible for membrane association and also for formation of punctate bodies.

Fig. 3. Images of BY-2 protoplasts expressing full-length or N-terminal portions of GRSPaV replicase polyprotein. Protoplasts were electroporated with various constructs and images were captured at 18 h post-electroporation. (a, b) pRTL2-GFP (positive control); (c, d) pREP-GFP expressing GFP-tagged full-length replicase polyprotein; (e, f) pREP1–749-GFP expressing the N-terminal 749 aa of the replicase polyprotein fused to GFP; (g, h) pREP1–207-GFP; (i, j) pREP1–131-GFP; (k, l) pREP132–207-GFP. Punctate bodies (indicated by arrows) were detected in protoplasts expressing REP-GFP (d), REP1–749-GFP (f), REP1–207-GFP (h) or REP132–207-GFP (l) but not in protoplasts expressing REP1–131-GFP (j). Scale bars, 20 μm.
Localization of GRSPaV replicase

A subdomain in the MTR determines the formation of punctate structures

To test if the MTR domain, specifically the putative amphipathic sequence identified above, was responsible for forming punctate bodies, we made three truncation constructs, pREP1–207-GFP, pREP1–131-GFP and pREP132–207-GFP, and investigated the subcellular distribution of each construct in BY-2 protoplasts. pREP1–207-GFP expresses a fusion protein where GFP is fused to the C terminus of a subdomain in the MTR domain of GRSPaV replicase polyprotein. A helical wheel projection of a lipid-binding amphipathic α-helix as predicted with HELIQUEST (Eisenberg et al., 1982; Gautier et al., 2008) is shown in (a), while the amino acid sequence of this putative membrane association region in relation to the replicase polyprotein is shown in (b). Note that this α-helix contains a polar face with multiple positively charged residues and a hydrophobic face (divided by the line). The polar face would be exposed to the cytoplasm whereas the hydrophobic face would reside in one leaflet of the lipid bilayer to achieve membrane association. Arrow in (a) depicts the direction of insertion of the amphipathic α-helix into cellular membrane. For explanation of protein symbols, see legend of Fig. 1.

The second construct, pREP1–131-GFP, expresses a GFP fusion containing a smaller portion of the MTR lacking the putative membrane-binding sequence. As shown in Fig. 3j, punctate bodies were no longer observed in protoplasts expressing this construct. Instead, diffuse green fluorescence, similar to the non-fused GFP, was observed throughout the cytoplasm (Fig. 3j, compare to Fig. 3b). Collectively, these data suggest that the region (aa 132–207) may contain sequence responsible for formation of the punctate bodies.

To further verify that the predicted IPM anchor sequence was indeed responsible for forming punctate bodies, we made a third construct, pREP132–207-GFP, which expresses a 76 aa region (aa 132–207) of the MTR including this putative amphipathic sequence. We designate this region a ‘subdomain’ to distinguish it from the shorter predicted membrane-binding sequence. Our choice to use a larger region than the putative amphipathic sequence segment (aa 132–207 versus 147–187) was based on the observation that a single copy of the amphipathic sequence from SFV, when fused to GFP, failed to target to the correct location (Spuul et al., 2007), presumably owing to the fact that the sequence was too short, which would interfere with protein targeting and membrane association. As shown in Fig. 3(i), we readily observed punctate bodies in protoplasts expressing REP132–207-GFP, which were similar to those produced in protoplasts expressing REP-GFP (Fig. 3d), REP1–749-GFP (Fig. 3f) and REP1–207-GFP (Fig. 3h). Similar structures were never observed in protoplasts transfected with pRTL2-GFP (Fig. 3b). Taken together, these results support the hypothesis that this subdomain within the MTR is responsible for the formation of punctate bodies in protoplasts.

Punctate structures formed by the N-terminal region of the replicase polyprotein appear to associate with ER

The size and shape of these punctate structures portrayed the possibility that they may be associated with one of several organelles. We knew that these structures were not associated with chloroplasts as demonstrated above (Fig. 2g–i). We then tested their possible association with two other organelles, Golgi and peroxisomes. To this end, we co-expressed pREP1–207-GFP with either pDsRED-ST (marker for Golgi; Samuels et al., 2007) or pRTL2-MFP-RFP (marker for peroxisome), followed by microscopic observations. It was clear that these punctate bodies co-localized neither with Golgi (Fig. 5a–c) nor with peroxisome (Fig. 5d–f). We then tested the possibility that these punctate structures might be associated with the ER. We co-expressed pREP1–207-GFP with an ER marker, pmRFP-ER (Rebelo et al., 2008), in BY-2 protoplasts. We observed that punctate bodies were located in close proximity to the ER network (Fig. 5g–i, I').
Membrane flotation and biochemical analyses confirm membrane association of the N-terminal region of GRSPaV replicase polyprotein

To obtain further evidence for membrane association of GRSPaV replicase polyprotein, we conducted subcellular fractionation and membrane flotation assays. *Agrobacterium tumefaciens* containing pREP1–207-GFP was infiltrated into *N. benthamiana* leaves. We also included, as controls, pER-GFP (expressing an ER-targeting GFP; Ju *et al.*, 2005) and pBIN35S : GUS-GFP (expressing a soluble protein, GUS-GFP; Xiao *et al.*, 2006). In preliminary experiments, only a few cells showed green fluorescence for pREP1–207-GFP after agro-infiltration (not shown). Consequently, the protein expression levels were too low to be detected. To enhance protein expression levels in all subsequent experiments, pREP1–207-GFP was co-infiltrated with pKyLx-p19 (Saxena *et al.*, 2011), expressing the strong suppressor of RNA silencing P19 of tomato bushy stunt virus (Voinnet *et al.*, 1999).

We first performed subcellular fractionations to test membrane association of these fusion proteins (Fig. 6a). The post-nuclear fractions (S3) derived from *N. benthamiana* leaves (2 days p.i.) expressing REP1–207-GFP, ER-GFP and GUS-GFP were subjected to high-speed centrifugation. The resulting supernatant (S30) and pellet (P30) were subjected to SDS-PAGE and Western blotting with anti-GFP antibody. As expected, GUS-GFP was detected mainly in the soluble S30 fraction, and ER-GFP in both S30 and membrane-enriched P30 fractions, while REP1–207-GFP was detected mainly in the P30 fractions (Fig. 6a), suggesting its association with membrane. It is worth noting that higher-order structures (dimer, trimer and oligomer/aggregate) were also detected in the P30 fractions of REP1–207-GFP (Fig. 6a). Similar phenomena were reported for many membrane

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Fig. 5. Images of BY-2 protoplasts co-expressing pREP1–207-GFP and markers for different organelles. Protoplasts were electroporated with a mixture of plasmids containing pREP1–207-GFP and Golgi marker pDsRed-ST (a–c), pREP1–207-GFP and peroxisome marker pMFP-RFP (d–f), or pREP1–207-GFP and ER marker pmRFP-ER (g–i). The punctate bodies formed by REP1–207-GFP do not appear to co-localize with the Golgi apparatus or peroxisome but are located in close proximity to the ER. A close-up image corresponding to the inset in (i) is shown at the bottom (I'). Images were taken 18 h post-electroporation. Scale bars, 20 μm.
proteins (Sanfaçon & Zhang, 2008). We also observed bands similar in size to ER-GFP in both the S30 and P30 fractions of REP$_{1-207}$-GFP (Fig. 6a, bottom, indicated by a bracket), possibly representing degradation products.

We then conducted membrane flotation to verify that REP$_{1-207}$-GFP in P30 fractions was due to membrane association and not simply aggregation resulting from overexpression. The P30 fractions of extracts from leaves infiltrated with pREP$_{1-207}$-GFP and pER-GFP were deposited individually on the bottom of a centrifuge tube, overlayed with a sucrose gradient and subjected to centrifugation at 100 000 g. The fractions were collected and subjected to SDS-PAGE/Western blotting. As shown in Fig. 6b, REP$_{1-207}$-GFP and ER-GFP were detected in fractions 8 and 9, towards the top of the gradient, which were expected to contain membrane-associated proteins. This confirms that both REP$_{1-207}$-GFP and ER-GFP were indeed associated with membrane. Note that there were also some proteins present in the bottom fractions (fractions 1 and 2), which, we believe, were due to protein aggregation.

To determine whether the fusion protein REP$_{1-207}$-GFP was an integral membrane protein, P30 fractions were extracted with 1 M NaCl or 0.1 M Na$_2$CO$_3$ (pH 11), followed by centrifugation at 30 000 g to separate solubilized protein (S) from membrane-bound protein (P). It is well established that treatment of P30 fractions with a high salt concentration (i.e. 1 M NaCl) allows the solubilization of peripheral membrane proteins, while integral membrane proteins remain associated with membrane. Under high-pH conditions, both peripheral and luminal proteins, but not integral membrane proteins, are released from membrane (Sanfaçon & Zhang, 2008). REP$_{1-207}$-GFP was not released from membrane after treatment with 1 M NaCl, and only a minute amount was released upon 0.1 M Na$_2$CO$_3$ treatment (Fig. 6c, left panel). This demonstrated that REP$_{1-207}$-GFP is an integral membrane protein. As expected, a small amount of ER-GFP was released after 1 M NaCl treatment, while treatment with 0.1 M Na$_2$CO$_3$, pH 11 released the majority of ER-GFP (Fig. 6c, right panel).

**DISCUSSION**

As a first step towards the elucidation of mechanisms of GRSPaV replication and infection, we investigated the subcellular localization and membrane association of the replicase polyprotein encoded by GRSPaV using two experimental systems. We found that the replicase polyprotein formed punctate bodies in N. benthamiana cells and tobacco BY-2 protoplasts. We demonstrated that the MTR domain, and specifically, a subdomain therein, contains sequences responsible for the generation of punctate structures. These punctate structures are not associated with chloroplasts, Golgi or peroxisomes, but are located in close proximity to the ER. Subcellular fractionation, membrane flotation and biochemical analyses demonstrate that the MTR domain associates with cellular membrane. However, the source of the membrane awaits identification.

The first step in the genome replication of (+)ssRNA viruses is the assembly of a VRC (Buck, 1996). It is known that VRCs are composed of virus-encoded replication-related proteins, viral RNAs and host factors. Furthermore, VRCs can anchor to a cellular membrane to form a specialized compartment, which protects viral RNA from degradation by host defence mechanisms, such as RNA silencing (David et al., 1992; Ahlquist et al., 2003; Ding et al., 2004; Mackenzie, 2005; Nagy & Pogany, 2008; Novoa et al., 2005; Waignmann et al., 2007). For example, the VRCs of TMV are macromolecular structures capable of replicating genomic and subgenomic RNAs, and contain viral replicase proteins, viral RNAs, and host proteins and membranes (Kawakami et al., 2004). These VRCs are given different names for different viruses: ‘spherules’ for BMV (Schwartz et al., 2002) and ‘cytoplasmic vacuoles’ for SFV (Kujala et al., 2001).

Most (+)ssRNA viruses studied to date form VRCs in association with the ER. Examples include polio-virus (Picornaviridae, Suhy et al., 2000), dengue virus (Flaviviridae, Welsch et al., 2009), BMV (Bromoviridae, Chen & Ahlquist, 2000), TMV (Virgaviroidae, dos Reis Figueira et al., 2002), PVX (Alphaflexiviridae, Bamunusinghe et al., 2009) and potato virus Y (Potyviridae; Wei & Wang, 2008). A few viruses recruit other types of intracellular membranes to form VRCs. For example, SFV forms VRCs on membranes of late endosomes/lysosomes (Froshauer et al., 1988; Kujala et al., 2001). Alfalfa mosaic virus and cucumber mosaic virus (both of the Bromoviridae) replicate in association with tonoplast membrane (Van Der Heijden et al., 2001; van der Heijden & Bol, 2002; Sztuba-Solinska & Bujarski, 2008). Most members of the Tombusviridae studied to date replicate in association with peroxisome membranes (Navarro et al., 2004, 2006; McCartney et al., 2005; Panavas et al., 2005; Jonczyk et al., 2007). Turnip yellow mosaic virus (TYMV, family Tombusviridae) replicates on chloroplast outer membrane (Prod’homme et al., 2001). It remains unknown as to what kinds of cellular membranes are utilized by viruses of the Betaflexiviridae. Considering that GRSPaV and TYMV both belong to Tombusvirales and the high levels of similarities in their replicase polyproteins, we first predicted that GRSPaV replicase would also target chloroplasts. However, the punctate bodies formed by GRSPaV replicase polyprotein co-localized neither with chloroplasts nor with Golgi or peroxisomes. Rather, they were located in close proximity to the ER. Membrane flotation and biochemical analyses demonstrate that the N-terminal 207 aa region of GRSPaV MTR associates with a cellular membrane. However, we cannot conclude at this point if the membrane was derived from the ER or other organelles, such as mitochondrion, endosome or tonoplast. Further research is needed to answer this question.

The association of virus replicase proteins with cellular membranes may occur in several ways. This can be
Fig. 6. Membrane flotation and biochemical analysis confirm membrane association of REP1–207-GFP from GRSPaV replicase. (a) Western blotting of subcellular fractions. Post-nuclear fractions derived from Agrobacterium-infiltrated N. benthamiana leaves (2 days p.i.) expressing REP1–207-GFP, ER-GFP and GUS-GFP were subjected to centriugation. The supernatant (S30) and pellet (P30) fractions were collected and subjected to SDS-PAGE and Western blotting with anti-GFP antibody. It can be seen that both REP1–207-GFP and ER-GFP are associated with cell membrane, as they floated to the top portions of the gradient after centrifugation. (c) Biochemical treatments of membrane-enriched fraction (P30) of N. benthamiana leaves infiltrated with A. tumefaciens containing pREP1–207-GFP (left) or pER-GFP (right). The P30 pellets were resuspended with 1 M NaCl or 0.1 M Na2CO3 (pH 11), followed by centrifugation at 30 000 g to separate the solubilized protein (S) released from the membrane-bound protein (P). Both the S and P fractions were subjected to SDS-PAGE and Western blotting with anti-GFP antibody. Molecular mass standards (in kDa) are provided as appropriate.

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METHODS

Expression constructs. As shown in Fig. 1, all constructs used for ectopic expression were made based on pRTL-2 (Restrepo et al., 1990) and three constructs were also subcloned in the mini binary vector pCB301(NotI) (Mann & Meng, 2013). To make pREP-GFP(RTL), site-directed mutagenesis (SDM) was used in conjunction with primers REPATAG(Bam)-F and REPATAG(Bam)-R to replace the stop codon of ORF1 with a BamHI site (Table 1, underlined) in the full-length viral clone pRSP28 (Meng et al., 2013). The eGFP sequence was amplified using primers GFFATATG(Bam)-F and GFFATATG(Bam)-R, amplifying the eGFP sequence without a start codon, a 3′ HA tag sequence (Table 1) and BamHI sites at both ends (Table 1). The amplified eGFP DNA was cloned into the above SDM product, producing pRSF.REP-GFP (not shown). The DNA sequence corresponding to REP-GFP was then subcloned into pRTL2 using Ncol and Xhol (Fig. 1).
To construct pREP1$_{1–131}$-GFP and pREP1$_{1–207}$-GFP, primer pairs MTF1 and MTR3, and MTF1 and MTR4 were used to amplify nucleotide positions 1–393 and 1–621 of ORF1, respectively, using pRSP28 as template. Primer MTF1 contained an Ncol site, followed by the sequence encoding the Myc tag (MEQKLISEED) and the 5'–terminal sequence of ORF1. The PCR products were then cloned into the Ncol site of pRTL2 to produce the intermediate constructs pREP1$_{1–131}$ and pREP1$_{1–207}$, which would express aa 1–131 and 1–207 of replicate polypeptide, respectively. To insert eGFP sequence at the 5' terminus of each intermediate construct, the 3' Ncol site was first replaced with Xbel site via SDM primer pairs SDM-MT1 and SDM-MT2, and SDM-MT3 and SDM-MT4. Subsequently, primer pairs GFPXbaF and GFPXbaR were used to amplify eGFP with flanking Xbel sites but without the start codon. Resulting PCR product was cloned into the Xbel site of each of the above SDM polypeptides, generating pREP1$_{1–131}$-GFP and pREP1$_{1–207}$-GFP (Fig. 1).

To produce pREP1$_{32–207}$-GFP, PCR was used to amplify a 3' portion of pREP1$_{3–207}$-GFP (from nt 456 to the stop codon of eGFP) with primers AmphNcoF and ctGFPstopSpeR. These primers incorporated an Ncol and a SpeI site at the 5' and 3' end of the amplified product, respectively. The PCR products were digested with Ncol and SpeI, and cloned into pRTL2 digested with Ncol and Xbel (with Xbel generating a site compatible with that of SpeI), generating pREP1$_{32–207}$-GFP (Fig. 1).

To produce pREP1$_{1–749}$-GFP, the region corresponding to aa 1–749 of the replicate polypeptide was amplified from pRSP28 using primers MTF1 and Rep749Xhr. Resulting PCR products were cloned into pRTL-2 after digestion with Ncol and Xbel. Subsequently, eGFP sequence was amplified using primers GFPXbaF and GFPXbaR, and cloned into the above construct digested with Xbel, resulting in pREP1$_{1–749}$-GFP.

**Agro-infiltration.** *N. benthamiana* plants at the five or six leaf stage were used for agro-infiltration. Two fully expanded leaves per plant were infiltrated on the abaxial side with *A. tumefaciens* strain EHA105 harbouring pREP-GFP$_{C(10)}$ adjusted to OD$_{600}$ 1. Procedures for *Agrobacterium* culture, induction and infiltration were as described in Meng et al. (2013).

**Protoplast isolation and electroporation.** Procedures for tobacco BY-2 cell culture, protoplast isolation and transfection were performed following the protocols of Rebelo et al. (2008) with the following modifications: (1) 25 ml 3-day-old BY-2 suspension cultures were used to isolate protoplasts; (2) $10^6$ protoplasts in 0.5 ml were mixed with 20 µg plasmid DNA for electroporation; and (3) electroporation was performed in a Gene Pulser Xcell (Bio-Rad) using one pulse at 250 V, 125 µF and 100 Ω.

**Fluorescence microscopy and CLSM.** Transfected epidermal cells of *N. benthamiana* leaves and BY-2 protoplasts were examined with a Leica DM4500B epifluorescence microscope (Leica Microsystems) equipped with Leica Application Suite (LAS) version 3.8. For GFP-tagged constructs, the GFP fluorophore was excited and emission collected with a Semrock BrightLine High Performance filter set FF506 (excitation peak at 482 nm, emission peak at 536 nm). For RFP-tagged constructs, the fluorescence was excited and emission collected with filter set FF562 (excitation peak at 531 nm, emission peak at 605 nm). To identify transfected protoplasts expressing different fusion proteins, protoplasts were first scanned at a magnification of ×100, followed by image capture at a magnification of ×400 or ×1000. For CLSM, Leica TCS SP5 (Leica Microsystems) equipped with an AFS imaging system was used. Green fluorescence was obtained at an excitation wavelength of 488 nm using an argon laser and emission wavelength of 500–520 nm. The red fluorescence of chloroplasts was obtained at an excitation wavelength of 488 nm and emission wavelength of 660–700 nm.

**Membrane flotation and biochemical treatment for membrane association.** *N. benthamiana* plants were infiltrated with *A. tumefaciens* EHA105 containing pREP1$_{3–207}$-GFP, pER-GFP, pBIN35S:GUS-GFP (Xiao et al., 2006) or pKyLx-P19 (Saxena et al., 2011). All Agrobacterium cultures were used were adjusted to OD$_{600}$ 1 with 10 mM MgCl$_2$. Agrobacterial cultures for pREP1$_{1–207}$-GFP, pER-GFP and pBIN35S:GUS-GFP were individually infiltrated, or co-infiltrated with pKyLx-P19 (Saxena et al., 2011). Procedures for Agrobacterium culture, induction and infiltration were as described in Meng et al. (2013). Subcellular fractionation, membrane flotation and biochemical treatment of membrane fractions were conducted.
essentially as described by Sanfaçon & Zhang (2008), with minor modifications. Agrobacterium-infiltrated N. benthamiana leaves (2 days p.i. for pREP1–207-GFP; 3 days p.i. for pER-GFP and pBIN355:GUS-GFP) were ground in three volumes of homogenization buffer using a mortar and pestle, and centrifuged at 3700 g for 10 min at 4 °C. The supernatant was collected as post-nuclear fractions (S3) and further centrifuged at 30 000 g for 20 min at 4 °C. The supernatant (S30) was collected and the pellet (membrane-enriched fraction, P30) was resuspended in a volume of homogenization buffer equal to that of the S30 fraction. Equal volumes of S30 and P30 fractions were subjected to SDS-PAGE and Western blotting. The P30 fractions were used for membrane flotation assay. Three hundred microlitres of P30 fractions were mixed with 1.6 ml 85% sucrose (final concentration of sucrose is 71.5%) and the mixture placed on the bottom of a 12 ml centrifuge tube (SW41; Beckman). The mixture was overlaid with 7 ml 65% sucrose and then 3.1 ml 10% sucrose. After centrifugation at 100 000 g for 18 h at 4 °C, 12 fractions of 1 ml each were collected from the top of the tube and subjected to SDS-PAGE and Western blotting.

To determine if proteins associate with membranes as integral or peripheral proteins, the P30 pellets were extracted with 0.1 M Na2CO3, and subjected to SDS-PAGE and Western blotting.

SDS-PAGE and Western blotting. SDS-PAGE and Western blotting were conducted as described by Sanfaçon & Zhang (2008). Protein samples were analysed on 8% (for pREP1–207-GFP) or 10% (for pER-GFP and pBIN355:GUS-GFP) SDS-PAGE gel and transferred onto PVDF membranes (Immobilon-P; Millipore). BLUeye Prestained Protein Ladder (FroggaBio) was used as molecular mass marker. A rabbit polyclonal anti-GFP antibody (Sigma) was used as the primary antibody and ECL donkey anti-rabbit IgG conjugated with horseradish peroxidase (GE Healthcare) as the secondary antibody. Signals were detected with CL-XPosure film (Thermo Scientific).

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