Sugarcane Mosaic Virus Remodels Multiple Intracellular Organelles to Form Genomic RNA Replication Sites

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Research Article

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Abstract

Positive-stranded RNA viruses usually remodel host endomembrane system to form virus-induced intracellular vesicles for replication during infections. The genus *Potyvirus* of *Potyviridae* represents the largest number of positive single-stranded RNA viruses and causes great damage on crop production worldwide. Though potyviruses have wide host ranges, each potyvirus infects relatively limited host species. Phylogenesis and host range analysis can divide potyviruses into monocot-infecting and dicot-infecting groups, suggesting that some infection mechanism, probably on replication may be distinct for each group. Comprehensive studies on the model dicot-infecting turnip mosaic virus indicated that the 6K2-induced replication vesicles are derived from endoplasmic reticulum (ER) and subsequently target chloroplasts for viral genome replication. However, we have no knowledge on the replication site of monocot-infecting potyviruses. In this study, we firstly show that the precursor 6K2-VPg-Pro polyproteins of dicot-infecting potyviruses and monocot-infecting potyviruses phylogenetically cluster in two separate groups. With a typical gramineae-infecting potyvirus sugarcane mosaic virus (SCMV), we found that SCMV replicative double-stranded RNA (dsRNA) forms aggregates in cytoplasm but does not associate with chloroplasts. SCMV 6K2-VPg-Pro-induced vesicles colocalize with replicative dsRNA. Moreover, SCMV 6K2-VPg-Pro-induced structures target multiple intracellular organelles including ER, Golgi apparatus, mitochondria and peroxisomes, and have no evident association with chloroplasts. In conclusion, SCMV remodels multiple intracellular organelles for its genomic RNA replication.

Introduction

During positive-strand RNA virus replication, the host endomembrane system is usually rearranged and contributes to the formation of virus-induced intracellular membranous vesicles providing scaffold for anchoring of virus replication complexes (VRCs) including viral proteins, RNA and host factors crucial for virus replication [1–3]. Diverse intracellular organelles or cellular comparts are subverted and modified by distinct viruses infecting plants and animals [4–6]. In particular, endoplasmic reticulum (ER) is widely targeted by various viruses to induce invagination of its outer membrane and form spherules or vesicles [7–13]. Besides ER, chloroplasts, mitochondria, peroxisomes, tonoplasts, nuclear membrane, and plasma membrane can also be rearranged to form membrane-bound VRCs in infected cells, indicating that these cellular membrane compartments are replication sites of corresponding viruses [14–19]. These membranous vesicles are thought to function in concentrating viral components and confine the virus replication in a specific compartment to escape host defense response and thereby to support the efficient replication of viruses [6, 20].

The genus *Potyvirus* contains agriculturally and economically important members causing great damage on many cultivated plant species including monocots and dicots [21, 22]. The potyviral genome is a positive-sense single-stranded RNA of approximately 10 kb in length that encodes a large polyprotein and subsequently being processed into 10 mature proteins [22–24]. Additionally, a P3N-PIPO resulted from viral RNA polymerase slippage is expressed within P3 cistron [25]. Of these viral proteins, 6K2 is an integral membrane protein that induces the formation of replication vesicles [8]. The 6K2-VPg-Pro

polyprotein of TuMV is demonstrated to be responsible for the induction of ER-derived cytoplasmic vesicles housing VRCs for virus replication by finding the presence of viral components of RNA-dependent RNA polymerase (RdRp), 6K1, P3, CI, VPg-Pro and double-stranded viral RNAs [26–30]. Various host factors are recruited to 6K2-VPg-Pro-induced replication vesicles for viral infection [31, 32], further suggesting that the 6K2-induced ER-derived vesicles are the sites for potyviral replication. However, potyviruses may also replicate in chloroplasts by evidences that viral RNAs of tobacco etch virus (TEV) and potato virus Y (PVY) were found in chloroplasts [33, 34]. Further studies precisely and clearly present evidence that 6K2-induced vesicles accumulate on ER membrane and migrate to Golgi apparatus, then target chloroplast envelope for TuMV genome replication [35, 36], indicating that both ER and chloroplasts are utilized for TuMV replication.

Previously, phylogenetic analysis of polyproteins encoded by potyviral genome sequences showed that monocot-infecting potyviruses clustered separately from that of dicot-infecting potyviruses [37, 38], suggesting a correlation between potyviral genomic sequences and host ranges. Considering this phylogenetic difference, the replication mechanisms of dicot- and monocot-infecting potyviruses may be distinct. Plenty of evidences have supported that both ER and chloroplasts are targeted by several dicot-infecting potyviruses for replication [7, 8, 33–35], while the replication sites for monocot-infecting potyviruses remain unclear.

Maize (*Zea mays*) is one of the most important and largest staple food crops in the world [39, 40]. The potyvirus sugarcane mosaic virus (SCMV) is a prevalent viral pathogen inducing maize dwarf mosaic disease worldwide [41, 42]. Beside maize, SCMV can infect several important monocot crops such as sorghum (*Sorghum vulgare*) and sugarcane (*Saccharum sinensis*) [41, 42]. Identification and characterization of the replication sites of SCMV would assist in precise understanding the virus replication process and its pathogenesis. In this study, we provide evidences that SCMV 6K2-VPg-Pro polyprotein-induced cytoplasmic vesicles are the virus replication sites that target multiple intracellular organelles.

Materials And Methods

Phylogenetic analysis

The amino acid sequences of potyviral 6K2-VPg-Pro were downloaded from the GenBank database in National Center for Biotechnology Information and then subjected to multiple sequence alignment and phylogenetic analysis using MAFFT online [43]. The phylogenetic tree was constructed with the neighbor-joining method. The phylogy was tested by 500 bootstrap resampling, poisson model amino acid substitutions type.

Plasmid construction

The coding regions of 6K2, NIa-VPg, and NIa-Pro were PCR-amplified using SCMV-BJ infectious clone as the template [44] with primers that were designed to modify the cleavage sites at the junction of 6K2 and

NIa-VPg, NIa-VPg and NIa-Pro to prevent polyprotein proteolysis (Supplementary Table 1) [45]. PCR products of 6K2, NIa-VPg and NIa-Pro were then fused together by overlap PCR to obtain 6K2-VPg-Pro, which was then inserted into pGD-mCherry and pGD-EGFP vectors [46] by Infusion enzyme (Takara, Kyoto, Japan) ligation to produce constructs pGD-6K2-VPg-Pro-mCherry and pGD-6K2-VPg-Pro-EGFP. The resulting constructs were confirmed by DNA sequencing.

Plant growth conditions and virus inoculation

Maize inbred line B73 and *Nicotiana benthamiana* plants were grown in growth champers under 16 h light at 24^{o}C/8 h dark at 22^{o}C conditions. The first true leaves of 8-day-old maize B73 seedlings were rub-inoculated with fresh crude extracts from plants infected by SCMV-BJ as previously described [47, 48]. Plants mock-inoculated with phosphate buffer (0.01 M) were used as controls.

Maize protoplasts isolation and transfection

Maize seeds were inoculated with fresh crude extracts from SCMV-BJ-infected leaves or mock-inoculated via vascular puncture, and the germinated seedlings were kept in dark at 24^{o}C to obtain etiolated plants [49, 50]. Maize protoplasts isolation and transfection were conducted as previously described [49, 51].

Particle bombardment assay

For particle bombardment assay, mock-inoculated or SCMV-BJ-infected maize plants which were sap-inoculated at 3-leaf-stage were allowed to grow to 9 leaf-stage in a greenhouse. The particle bombardment assay was performed as described previously with some modifications [52]. The prepared microcarriers (50 µL) were mixed with 2.5 µg plasmids, 20 µL of 0.1 M spermidine and 50 µL of 2.5 M $CaCl_2$. After vortex for 3 min, the resulting mixture was centrifuged at 10000 g for 20 s. The pellet was resuspended and washed with 140 µL 70% ethanol twice. Finally, the pellet was resuspended by 10 µL absolute ethanol for bombardment. The bombardment assay was performed with a PDS-1000/He system (Bio-Rad) as instructions. Tissue helium pressure at the tank was regulated at 1,500 pounds per square inch. The bombarded maize leaf tissues were kept in dark conditions at 25^{o}C for 18-24 h until confocal microscopy analysis.

Agrobacterium-mediated transient expression in *N. benthamiana*

Plasmids for agroinfiltration were introduced into *Agrobacterium tumefaciens* strain GV3101 by freeze-thaw method [53]. The bacteria were then cultured overnight, centrifuged to obtain pellet, and finally resuspended with MMA buffer containing 10 mM $MgCl_2$, 10 mM MES, pH 5.6, and 100 mM acetosyringone. The resuspended bacteria were then diluted to OD_{600}=0.5, followed by incubation at room temperature for 2-3 h until infiltration into the abaxial face of leaves of 5- or 6-leaf-stage *N. benthamiana* plants. To enhance protein expression, agrobacteria harboring a plasmid expressing the RNA silencing suppressor p19 protein of tomato bushy stunt virus (TBSV) were also infiltrated simultaneously [54].

Confocal microscopy analysis

Agroinfiltrated *N. benthamiana* leaves, transfected maize protoplasts and bombarded leaf tissues were analyzed using a confocal laser-scanning microscopy (Leica TCS SP8). The fluorescence of both EGFP and YFP was excited at 488 nm, mCherry and chloroplasts autofluorescence was excited at 552 and 638 nm, respectively. A line sequential scanning mode was used to avoid fluorescence across.

Western blot analysis

Total protein of maize leaf tissues and protoplasts were extracted as previously described [48]. The protein extracts were separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, followed by transferring proteins to PVDF membranes (Millipore, Massachusetts, USA). The blotted membranes were then separately incubated with anti-SCMV CP [55] and anti-actin (CW0264, CWBIO, Beijing, China) antibodies with a dilution of 1:5000 for 1 h, and finally detected chemiluminescence with eECL Western Blot Kit according to manufacturer's protocol (CW0049, CWBIO, Beijing, China).

Results

6K2-VPg-Pro polyproteins of monocot-infecting potyviruses phylogenetically separate from that of dicot-infecting potyviruses

To investigate whether the replication site of monocot-infecting potyviruses is same or distinct to that of dicot-infecting potyviruses, we firstly conducted a multiple sequence alignment of potyviral 6K2-VPg-Pro polyprotein amino acid sequences (Supplementary Fig. 1) and performed a phylogenetic analysis (Fig. 1) using the neighbor-joining method implemented with MAFFT online [43]. Multiple sequences alignment showed that the 6K2-VPg-Pro polyproteins share 58.55% identities among the representative 20 potyviruses, and there are 54 absolutely conserved residues for all of them (Supplementary Fig. 1). Strikingly, the 39W, 299N, 413S residues were relatively conserved in dicot-infecting potyviruses, whereas the three sites in monocot-infecting potyviruses were mostly conserved as 39L, 299P, 413D (Supplementary Fig. 1). The phylogenetic tree showed two main clades including a monocot-infecting cluster and a dicot-infecting potyviruses cluster (Fig. 1). In detail, clade 1 contained only monocot-infecting potyviruses including pennisetum mosaic virus (PenMV), maize dwarf mosaic virus (MDMV), SCMV, sorghum mosaic virus (SrMV), johnsongrass mosaic virus (JGMV), and cocksfoot streak virus (CSV), and clade 2 only consists of dicot-infecting potyviruses (Fig. 1).

SCMV replication sites do not correlate with chloroplasts

Double-stranded RNA (dsRNA) is regarded as the hallmark of virus infection [56]. For positive single-stranded RNA viruses, replicative RNA intermediates, namely viral dsRNA is formed during virus replication, whose localization is reasonably thought to be the site of virus replication. To label the replication sites of SCMV in maize cells, we performed the dsRNA binding-dependent fluorescence complementation (dRBFC) assay by co-expressing B2-nYFP and VP35-cYFP in SCMV-infected maize

cells following a previous report [36]. In this assay, maize protoplasts isolated from mock- or SCMV-systemically infected leaves (see methods) were transfected with plasmids expressing B2-nYFP and VP35-cYFP. Confocal microscopic analysis showed that fluorescent punctate structures indicating SCMV dsRNA localization were observed in cytoplasm of SCMV-infected maize protoplasts (Fig. 2A), and the reconstituted YFP signals have no obvious association with chloroplasts autofluorescence (Fig. 2A). No fluorescent signal was observed in mock-infected maize protoplasts (Fig. 2A). Western blotting confirmed the infection of SCMV in maize protoplasts (Supplementary Fig. 2A).

Moreover, we performed the dRBFC assay in maize leaves by particle bombardment. The upper maize leaves were collected from healthy and SCMV-systemically infected plants at 9-leaf stage, and subjected to bombardment. The dsRNA-reconstituted YFP fluorescent signals were observed in cytoplasm but did not merge with the red autofluorescence of chloroplasts in SCMV-systemically infected maize leaf cells (Fig. 2B). No YFP fluorescence was observed in healthy leaf cells. Western blotting confirmed the infection of SCMV in maize leaves for bombardment (Supplementary Fig. 2B).

The 6K2-VPg-Pro-induced cytoplasmic vesicles are replication sites of SCMV

Studies on dicot-infecting potyviruses, such as TEV and TuMV, demonstrated that the 6K2-VPg-Pro polyprotein can induce the formation of cytoplasmic vesicles, which were further shown to be their replication sites [8, 26, 35]. To determine whether SCMV-encoded 6K2-VPg-Pro could induce cytoplasmic vesicle structures to function as virus replication sites, we performed the dRBFC assay by co-expressing B2-nYFP and VP35-cYFP with 6K2-VPg-Pro in SCMV-infected maize leaves. To this end, 6K2-VPg-Pro was tagged with mCherry at C-terminal (Fig. 3A). It should be noted that the Glutamine (Q) and Glutamic acid (E) residues preceding the cleavage sites locate at 6K2-VPg and VPg-NIa-Pro junctions were both changed by histidine (H), thereby preventing protein proteolysis [45]. We then conducted a particle bombardment assay in SCMV-infected maize leaves, in which mCherry-tagged 6K2-VPg-Pro (6K2-VPg-Pro-mCherry) was co-expressed with B2-nYFP and VP35-cYFP. The results showed that granular YFP foci formed by dsRNA binding of B2-nYFP and VP35-cYFP perfectly overlapped with the irregular structures induced by 6K2-VPg-Pro-mCherry (Fig. 3B), suggesting that the 6K2-VPg-Pro-induced irregular structures are the active replication sites of SCMV in maize leaf cells.

The 6K2-VPg-Pro-induced replication vesicles reside on multiple intracellular organelles excluding on chloroplasts

Considering that the TuMV 6K2-induced replication vesicles migrate sequentially from ER to chloroplasts for viral genome replication [35], we wondered whether SCMV could also target chloroplasts for replication. For this test, we made a construct 6K2-VPg-Pro-EGFP by fusing EGFP to the C-terminal of 6K2-VPg-Pro (Fig. 4A), and delivered it into protoplasts isolated from SCMV-systemically infected maize leaves. Comparing with the nucleus and cytoplasm-localized wild EGFP, aggregate structures showing green fluorescence could only be observed in cytoplasm of protoplasts transfected with 6K2-VPg-Pro-EGFP (Fig. 4B). Unexpectedly, these structures clearly had no obvious association with the red autofluorescence from chloroplasts (Fig. 4B). Moreover, in *N. benthamiana* epidermal cells, transient

expression of either 6K2-VPg-Pro-EGFP or 6K2-VPg-Pro-mCherry also formed aggregate bodies and punctate structures in cytoplasm (Supplementary Fig. 3). These aggregate bodies and punctate structures did not merge with the chloroplasts autofluorescence (Supplementary Fig. 3), suggesting that SCMV 6K2-VPg-Pro-induced replication vesicles did not reside on chloroplasts.

Previous findings that TuMV 6K2 vesicles accumulate at ER exit sites (ERES) on the ER membrane and subsequently migrate to Golgi apparatus [35, 36] prompted us to investigate whether SCMV 6K2-VPg-Pro-induced vesicles locate at ER and/or Golgi apparatus. We co-expressed 6K2-VPg-Pro-EGFP with an ER marker mCherry-HDEL or a Golgi apparatus marker mCherry-GmMan1 in maize protoplasts [57]. Expectedly, SCMV 6K2-VPg-Pro-EGFP-induced punctate structures colocalized with mCherry-HDEL and mCherry-GmMan1 (Fig. 4C, D), demonstrating that SCMV replicates at both ER and Golgi apparatus. Similar co-localizations were also observed when expressed in *N. benthamiana* leaf epidermal cells (Fig. 4C, D).

We further tempted to determine whether SCMV 6K2-VPg-Pro could target other organelles such as mitochondria and peroxisomes. Intriguingly, we found that 6K2-VPg-Pro-EGFP-induced punctate structures merged with mCherry-ScCOX4 [57] and peroxisome marker dsRed-SKL [58] in *N. benthamiana* leaf epidermal cells (Supplementary Fig. 4). Taken together, for replication SCMV can target multiple intracellular organelles including ER, Golgi apparatus, mitochondria and peroxisomes but not chloroplasts.

Discussion

In this study, we investigate the active replication sites of SCMV in maize cells through dRBFC assay. As a sensitive and specific dsRNA reporter system for visualizing dsRNA distribution and dynamics in living cells, dRBFC has been used *in vivo* to visualize the subcellular distribution of dsRNA intermediates in the replication of TuMV, carnation Italian ringspot virus, barley stripe mosaic virus [36, 59]. Given that dRBFC assay requires the active viral replication which generates double-stranded replicative RNA intermediates [36], we used young maize leaves already systemically infected with SCMV for protoplasts preparation and bombardment to locate the replication sites of SCMV. The systemic infection of SCMV in young leaves ensures the active replication state of SCMV in maize cells. The dRBFC assay clearly showed that SCMV replicative dsRNA localized in cytoplasm but did not reside on chloroplasts in maize protoplasts and leaf tissues.

We then demonstrated that SCMV replicative dsRNA associated with 6K2-VPg-Pro-induced vesicles. SCMV-encoded 6K2-VPg-Pro polyprotein is responsible for the formation of vesicles represented by irregular punctate and aggregated structures in cytoplasm, which is consistent with that the expression of TuMV 6K2-VPg-Pro could induce the formation of cytoplasmic vesicles [26]. The ring-like vesicle structures induced by SCMV 6K2-VPg-Pro were similar to that of vesicle structures induced by TuMV 6K2 [35]. These punctate or aggregated bodies may come from the membrane-binding and fusion effects of these vesicles [8]. Among the SCMV 6K2-VPg-Pro-induced vesicles, some perfectly colocalized to dsRNA

replicative intermediates indicating virus replication, while some scattered in cytoplasm rather than colocalized with dsRNA (Fig. 3B). Since the co-localization of 6K2-VPg-Pro-induced vesicles with dsRNA binding protein (B2-YN and VP35-YC), the SCMV 6K2-VPg-Pro-induced vesicles can provide compartments for SCMV active RNA replication.

Studies on TuMV 6K2 revealed it can induce small mobile vesicles derived from ER at early infection stage and form an irregularly shaped structure juxtaposed to nucleus at late infection stages [8]. TuMV RNA replication takes place within these 6K2 vesicles associating with chloroplasts or in cytoplasm [8, 27, 35, 36]. TuMV 6K2-induced replication vesicles form at ERES on ER membrane, and then transport to Golgi apparatus by COPI and COPII secretory pathway, and subsequently reside on chloroplasts for virus replication. Viral RNA was also detected in chloroplasts [7, 8, 33–35, 60]. Even in the absence of other viral proteins/viral replication, TuMV 6K2 can induce the biogenesis of ER-derived vesicles that target chloroplasts in *N. benthamiana* leaves [36, 60]. However, for SCMV 6K2-derived vesicles, we only observed them in cytoplasm. We did not find SCMV 6K2-derived vesicles targeting chloroplasts in maize protoplasts or bombarded epidermal cells.

In this study, SCMV 6K2-VPg-Pro-induced vesicles were firstly found to be associated with ER and Golgi apparatus, while had no evident co-localization with chloroplasts. We wondered whether other intracellular organelles can be employed by SCMV as replication sites. Interestingly, SCMV 6K2-VPg-Pro-EGFP-formed punctate structures also colocalized with both mitochondria and peroxisome markers in *N. bethamiana* leaf epidermal cells, which was similar to the replication sites of a well-studied tombusvirus TBSV [61, 19]. TBSV employs peroxisomes and mitochondria to form VRCs, and can target ER for replication when peroxisome biogenesis is defective, implying its preference in targeting organelles for replication [19, 35, 61]. It would be interesting to explore whether SCMV is preferential in remodeling organelles to form VRCs by using organelles biogenesis-defective maize plants under infection condition.

A question is which mechanisms exist underlying the difference of replication sites between monocot-infecting and dicot-infecting potyviruses. Previous studies showed that polyproteins encoded by monocot-infecting and dicot-infecting potyviruses cluster separately in distinct subgroups [37, 38]. Genetic variation and phylogenetic analysis revealed that the 6K2-VPg-Pro polyproteins of monocot-infecting potyviruses cluster separately with that of dicot-infecting potyviruses. There are 54 conserved residues in 6K2-VPg-Pro of monocot- and dicot-infecting potyviruses, suggesting their important function in potyviral replication. Intriguingly, we found the combination of three relatively conserved amino acids at positions 39, 299 and 413 between monocot- and dicot-infecting potyviruses clusters, that are W^{39}, N^{299} and S^{413} for dicot-infecting potyviruses, while L^{39}, P^{299} and D^{413} for monocot-infecting potyviruses. The genetic variations on 6K2-VPg-Pro between monocot- and dicot-infecting potyviruses potentially suggest their roles in determining the replication sites. TuMV 6K2-induced vesicles can traffic to chloroplasts by relying on actomyosin motility system [35]. As for monocot-infecting potyviruses like SCMV, we propose that actomyosin motility system or other chloroplast transport pathway may not be adopted by 6K2-VPg-Pro. Future work on genetic variations determining the distinct characteristics of

replication vesicles will provide clues for the differences on replication sites and host range between monocot-infecting and dicot-infecting potyviruses.

Declarations

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Authors' contributions

J.X. and T.Z. designed the experiments. J.X. performed most experiments. J.X., T.J., Z.L., X.L., Z.F. and T.Z. analyzed the data. J.X. and T.Z. wrote the manuscript. All authors read and approved the final manuscript.

Conflicts of interest

The authors declare that they have no conflict of interest.

Ethics approval

This article does not contain any studies with human participants or animals performed by any of the authors.

References

1. Ahlquist P, Noueiry AO, Lee WM, Kushner DB, Dye BT (2003) Host factors in positive-strand RNA virus genome replication. J Virol 77:8181–8186.
2. Belov GA, van Kuppeveld FJM (2012) (+)RNA viruses rewire cellular pathways to build replication organelles. Curr Opin Virol 2:740–747.

3. Nagy PD, Pogany J (2011) The dependence of viral RNA replication on co-opted host factors. Nat Rev Microbiol 10:137–149.
4. Cho MW, Teterina N, Egger D, Bienz K, Ehrenfeld E (1994) Membrane rearrangement and vesicle induction by recombinant poliovirus 2C and 2BC in human cells. Virology 202:129–145.
5. Barco A, Carrasco L (1995) Human virus protein, poliovirus protein 2bc, induces membrane proliferation and blocks the exocytic pathway in the yeast *Saccharomyces Cerevisiae*. EMBO J 14:3349–3364.
6. Laliberté JF, Sanfaçon H (2010) Cellular remodeling during plant virus infection. Annu Rev Phytopathol 48:69–91.
7. Martin MT, Cervera MT, Garcia JA, Bonay P (1995) Properties of the active plum pox potyvirus RNA polymerase complex in defined glycerol gradient fractions. Virus Res 37:127–137.
8. Schaad MC, Jensen PE, Carrington JC (1997) Formation of plant RNA virus replication complexes on membranes: role of an endoplasmic reticulum-targeted viral protein. EMBO J 16:4049–4059.
9. Carette JE, Stuiver M, van Lent J, Wellink J, van Kammen A (2000) Cowpea mosaic virus infection induces a massive proliferation of endoplasmic reticulum but not Golgi membranes and is dependent on de novo membrane synthesis. J Virol 74:6556–6563.
10. Suhy DA, Giddings TH, Kirkegaard K (2000) Remodeling the endoplasmic reticulum by poliovirus infection and by individual viral proteins: an autophagy-like origin for virus-induced vesicles. J Virol 74:8953–8965.
11. Schwartz M, Chen J, Janda M, Sullivan M, den Boon J, Ahlquist P (2002) A positive-strand RNA virus replication complex parallels form and function of retrovirus capsids. Mol Cell 9:505–514.
12. Nishikiori M, Dohi K, Mori M, Meshi T, Naito S, Ishikawa M (2006) Membrane-bound tomato mosaic virus replication proteins participate in RNA synthesis and are associated with host proteins in a pattern distinct from those that are not membrane bound. J Virol 80:8459–8468.
13. Bamunusinghe D, Hemenway CL, Nelson RS, Sanderfoot AA, Ye CM, Silva MAT et al (2009) Analysis of potato virus X replicase and TGBp3 subcellular locations. Virology 393:272–285.
14. Hatta T, Bullivant S, Matthews RE (1973) Fine structure of vesicles induced in chloroplasts of Chinese cabbage leaves by infection with turnip yellow mosaic virus. J Gen Virol 20:37–50.
15. Weber-Lotfi F, Dietrich A, Russo M, Rubino L (2002) Mitochondrial targeting and membrane anchoring of a viral replicase in plant and yeast cells. J Virol 76:10485–10496.
16. Hagiwara Y, Komoda K, Yamanaka T, Tamai A, Meshi T, Funada R et al (2003) Subcellular localization of host and viral proteins associated with tobamovirus RNA replication. EMBO J 22:344–353.
17. Pouwels J, van der Velden T, Willemse J, Borst JW, van Lent J, Bisseling T et al (2004) Studies on the origin and structure of tubules made by the movement protein of cowpea mosaic virus. J Gen Virol 85:3787–3796.

18. Goodin M, Yelton S, Ghosh D, Mathews S, Lesnaw J (2005) Live-cell imaging of rhabdovirus-induced morphological changes in plant nuclear membranes. Mol Plant-Microbe Interact 18:703–709.
19. McCartney AW, Greenwood JS, Fabian MR, White KA, Mullen RT (2005) Localization of the tomato bushy stunt virus replication protein p33 reveals a peroxisome-to-endoplasmic reticulum sorting pathway. Plant Cell 17:3513–3531.
20. Wileman T (2006) Aggresomes and autophagy generate sites for virus replication. Science 312:875–878.
21. Ivanov KI, Eskelin K, Lõhmus A, Mäkinen K (2014) Molecular and cellular mechanisms underlying potyvirus infection. J Gen Virol 95:1415–1429.
22. Revers F, García JA (2015) Molecular biology of potyviruses. Adv Virus Res 92:101–199.
23. Urcuqui-Inchima S, Haenni AL, Bernardi F (2001) Potyvirus proteins: a wealth of functions. Virus Res 74:157–175.
24. Chung BYW, Miller WA, Atkins JF, Firth AE (2008) An overlapping essential gene in the Potyviridae. Proc Natl Acad Sci U S A 105:5897–5902.
25. Olspert A, Chung BYW, Atkins JF, Carr JP, Firth AE (2015) Transcriptional slippage in the positive-sense RNA virus family *Potyviridae*. EMBO Rep 16: 995-1004.
26. Beauchemin C, Boutet N, Laliberté JF (2007) Visualization of the interaction between the precursors of VPg, the viral protein linked to the genome of turnip mosaic virus, and the translation eukaryotic initiation factor iso 4E in planta. J Virol 81:775–782.
27. Cotton S, Grangeon R, Thivierge K, Mathieu I, Ide C, Wei T et al (2009) Turnip mosaic virus RNA replication complex vesicles are mobile, align with microfilaments, and are each derived from a single viral genome. J Virol 83:10460–10471.
28. Cui X, Wei T, Chowda-Reddy RV, Sun G, Wang A (2010) The tobacco etch virus P3 protein forms mobile inclusions via the early secretory pathway and traffics along actin microfilaments. Virology 397:56–63.
29. Wan J, Basu K, Mui J, Vali H, Zheng H, Laliberté JF (2015) Ultrastructural characterization of turnip mosaic virus-induced cellular rearrangements reveals membrane-bound viral particles accumulating in vacuoles. J Virol 89:12441–12456.
30. Cui H, Wang A (2016) Plum pox virus 6K1 protein is required for viral replication and targets the viral replication complex at the early stage of infection. J Virol 90:5119–5131.
31. den Boon JA, Diaz A, Ahlquist P (2010) Cytoplasmic viral replication complexes. Cell Host & Microbe 8:77–85.
32. Wang A (2015) Dissecting the molecular network of virus-plant interactions: the complex roles of host factors. Annu Rev Phytopathol 53:45–66.
33. Gadh IP, Hari V (1986) Association of tobacco etch virus related RNA with chloroplasts in extracts of infected plants. Virology 150:304–307.

34. Gunasinghe UB, Berger PH (1991) Association of potato virus-Y gene-products with chloroplasts in tobacco. Mol Plant-Microbe Interact 4:452–457.
35. Wei T, Huang TS, McNeil J, Laliberté JF, Hong J, Nelson RS et al (2010) Sequential recruitment of the endoplasmic reticulum and chloroplasts for plant potyvirus replication. J Virol 84:799–809.
36. Cheng X, Deng P, Cui HG, Wang A (2015) Visualizing double-stranded RNA distribution and dynamics in living cells by dsRNA binding-dependent fluorescence complementation. Virology 485:439–451.
37. Gibbs A, Ohshima K (2010) Potyviruses and the digital revolution. Annu Rev Phytopathol 48:205–223.
38. Moury B, Desbiez C (2020) Host range evolution of potyviruses: A global phylogenetic analysis. Viruses 12:111.
39. Pechanova O, Pechan T (2015) Maize-pathogen interactions: an ongoing combat from a proteomics perspective. Int J Mol Sci 16:28429–28448.
40. Zuo W, Chao Q, Zhang N, Ye J, Tan G, Li B et al (2015) A maize wall-associated kinase confers quantitative resistance to head sm Nat Genet 47:151–157.
41. Shukla DD, Tosic M, Jilka J, Ford RE, Toler RW, Langham MAC (1989) Taxonomy of potyviruses infecting maize, sorghum, and sugarcane in Australia and the United States as determined by reactivities of polyclonal antibodies directed towards virus-specific N-termini of coat proteins. Phytopathology 79:223–229.
42. Xiao XW, Frenkel MJ, Teakle DS, Ward CW, Shukla DD (1993) Sequence diversity in the surface-exposed amino-terminal region of the coat proteins of seven strains of sugarcane mosaic virus correlates with their host range. Arch Virol 132:399–408.
43. Katoh K, Rozewicki J, Yamada KD (2019) MAFFT online service: multiple sequence alignment, interactive sequence choice and visualization. Brief Bioinform 20:1160–1166.
44. Liu W (2015) Studies on the construction strategy of infectious clones of Sugarcane mosaic virus. Dissertation, China Agricultural University, Beijing.
45. Laliberté JF, Nicolas O, Chatel H, Lazure C, Morosoli R (1992) Release of a 22-kDa protein derived from the amino-terminal domain of the 49-kDa Nla of turnip mosaic potyvirus in *Escherichia coli*. Virology 190:510–514.
46. Goodin MM, Dietzgen RG, Schichnes D, Ruzin S, Jackson AO (2002) pGD vectors: versatile tools for the expression of green and red fluorescent protein fusions in agroinfiltrated plant leaves. Plant J 31: 375-383.
47. Fan ZF, Chen HY, Liang XM, Li HF (2003) Complete sequence of the genomic RNA of the prevalent strain of a potyvirus infecting maize in China. Arch Virol 148: 773-782.
48. Cao Y, Shi Y, Li Y, Cheng Y, Zhou T, Fan Z (2012) Possible involvement of maize Rop1 in the defence responses of plants to viral infection. Mol Plant Pathol 13:732–743.
49. Sheen J (1991) Molecular mechanisms underlying the differential expression of maize pyruvate, orthophosphate dikinase genes. Plant Cell 3:225–245.

50. Wang R, Yang X, Wang N, Liu X, Nelson RS, Li W et al (2016) An efficient virus-induced gene silencing vector for maize functional genomics research. Plant J 86:102–115.
51. Yoo SD, Cho YH, Sheen J (2007) Arabidopsis mesophyll protoplasts: a versatile cell system for transient gene expression analysis. Nat Protoc 2:1565–1572.
52. Kirienko DR, Luo A, Sylvester AW (2012) Reliable transient transformation of intact maize leaf cells for functional genomics and experimental study. Plant Physiol 159:1309–1318.
53. Holsters M, de Waele D, Depicker A, Messens E, van Montagu M, Schell J (1978) Transfection and transformation of *Agrobacterium tumefaciens*. Mol Gen Genet 163:181–187.
54. Qu F, Morris TJ (2002) Efficient infection of *Nicotiana benthamiana* by Tomato bushy stunt virus is facilitated by the coat protein and maintained by p19 through suppression of gene silencing. Mol Plant Microbe Interact 15: 193-202.
55. Chen H, Cao H, Li Y, Xia Z, Xie J, Carr JP et al (2017) Identification of differentially regulated maize proteins conditioning *Sugarcane mosaic virus* systemic infection. New Phytol 215: 1156-1172.
56. Jacobs BL, Langland JO (1996) When two strands are better than one: The mediators and modulators of the cellular responses to double-stranded RNA. Virology 219:339–349.
57. Nelson BK, Cai X, Nebenführ A (2007) A multicolored set of in vivo organelle markers for co-localization studies in Arabidopsis and other plants. Plant J 51: 1126-1136.
58. Li F, Zhao N, Li Z, Xu X, Wang Y, Yang X et al (2017) A calmodulin-like protein suppresses RNA silencing and promotes geminivirus infection by degrading SGS3 via the autophagy pathway in *Nicotiana benthamiana*. PLoS Pathog 13: e1006213
59. Zhang K, Zhang Y, Yang M, Liu S, Li Z, Wang X et al (2017) The barley stripe mosaic virus γ protein promotes chloroplast-targeted replication by enhancing unwinding of RNA duplexes. PLoS Pathog 13: e1006319.
60. Wei T, Wang A (2008) Biogenesis of cytoplasmic membranous vesicles for plant potyvirus replication occurs at endoplasmic reticulum exit sites in a COPI- and COPII-dependent manner. J Virol 82:12252–12264.
61. Jonczyk M, Pathak KB, Sharma M, Nagy PD (2007) Exploiting alternative subcellular location for replication: tombusvirus replication switches to the endoplasmic reticulum in the absence of peroxisomes. Virology 362: 320-330.

Figures

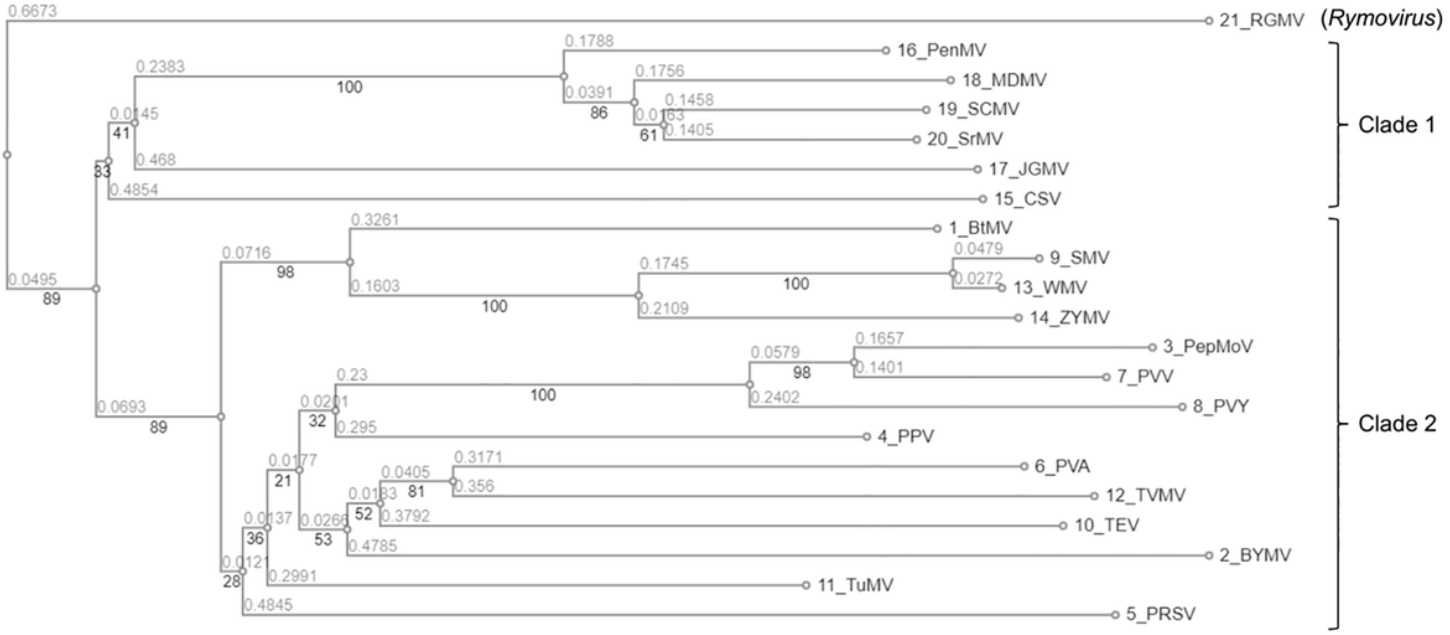

Figure 1

Phylogenetic analysis based on 6K2-VPg-Pro amino acid sequences of the 20 representative potyviruses. The phylogenetic tree was constructed with MAFFT online [43]. The confidence values are highlight in bold and shown below the gray lines, the branch length values are shown in gray and stay above each line. The name of potyviruses and their GenBank accession numbers are shown as follows: pennisetum mosaic virus (PenMV), NC_007147.1; maize dwarf mosaic virus (MDMV), NC_003377.1; sugarcane mosaic virus (SCMV), NC_003398.1; sorghum mosaic virus (SrMV), NC_004035.1; johnsongrass mosaic virus (JGMV), NC_003606.1; cocksfoot streak virus (CSV), NC_003742.1; beet mosaic virus (BtMV), NC_005304.1; soybean mosaic virus (SMV), NC_002634.1; watermelon mosaic virus (WMV), NC_006262.1; zucchini yellow mosaic virus (ZYMV), NC_003224.1; pepper mottle virus (PepMoV), NC_001517.1; potato virus V (PVV), NC_004010.1; potato virus Y (PVY), NC_001616.1; plum pox virus (PPV), NC_001445.1; potato virus A (PVA), NC_004039.1; tobacco vein mottling virus (TVMV), NC_001768.1; tobacco etch virus (TEV), NC_001555.1; bean yellow mosaic virus (BYMV), NC_003492.1; turnip mosaic virus (TuMV), NC_002509.2; papaya ringspot virus (PRSV), NC_001785.1. Note that monocot-infecting potyviruses including PenMV, MDMV, SCMV, SrMV, JGMV and CSV cluster into Clade 1, while dicot-infecting potyviruses including BtMV, SMV, WMV, ZYMV, PepMoV, PVV, PVY, PPV, PVA, TVMV, TEV, BYMV, TuMV, PRSV cluster into Clade 2. Ryegrass mosaic virus (RGMV, NC_001814.1), a member of genus Rymovirus in family Potyviridae, was used as an outgroup.

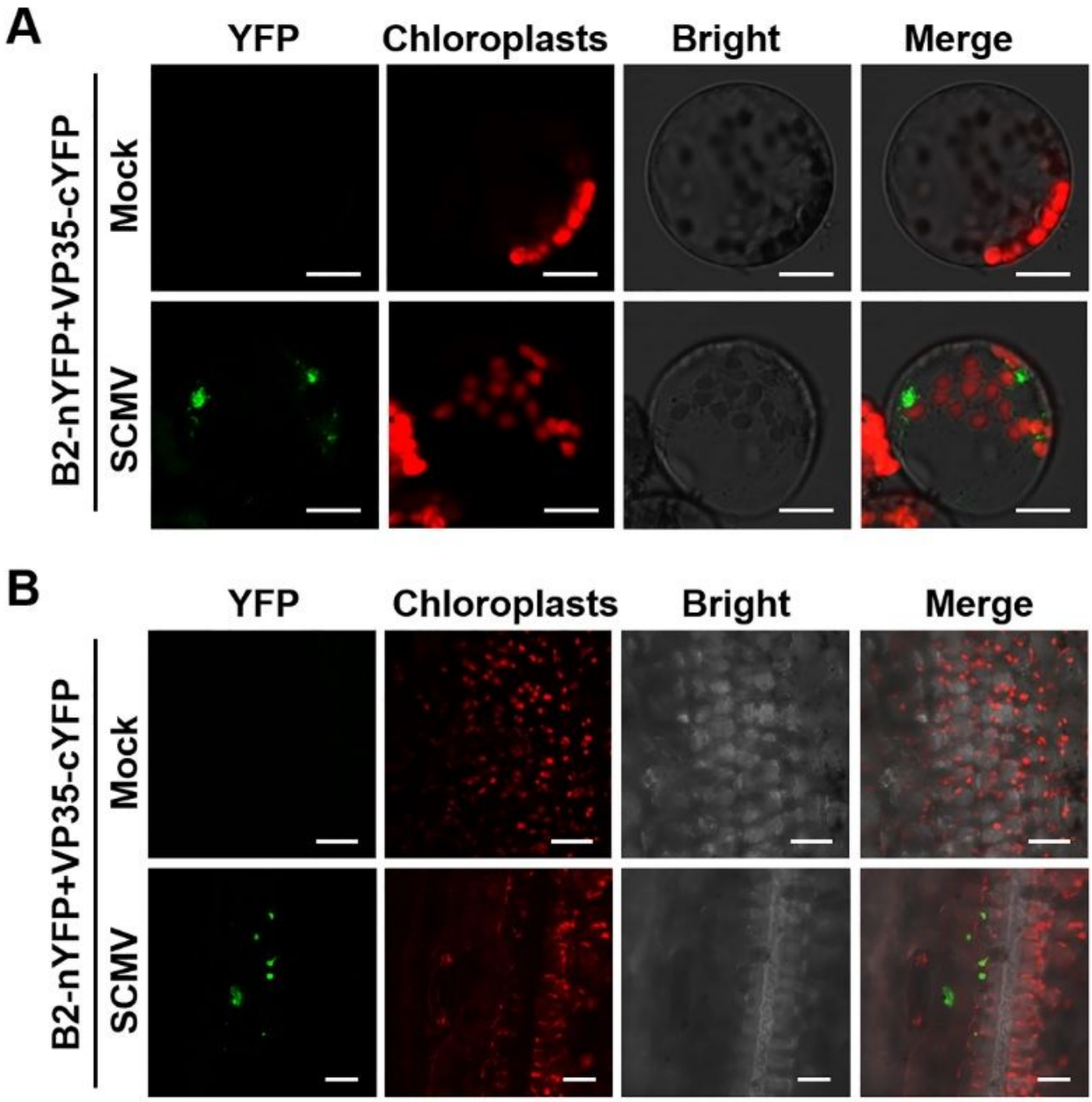

Figure 2

SCMV replicative dsRNA does not associate with chloroplasts. (A) SCMV replicative dsRNA has no association with chloroplasts in maize protoplasts. Plasmids containing split B2-nYFP and VP35-cYFP were co-delivered into protoplasts prepared from Mock- or SCMV-infected maize leaves. YFP signal was be observed in SCMV-infected maize protoplasts at 20 hours post transfection (hpt) by confocal microscope. Chloroplast autofluorescence is displayed in red. Bars=10 μm. (B) SCMV replicative dsRNA does not localize at chloroplasts in maize leaf tissues. Plasmids expressing split B2-nYFP and VP35-cYFP were bombarded into Mock-inoculated and SCMV-infected maize leaf cells. Leaf tissues were

subjected to confocal microscopic analysis at 20 hours post bombardment (hpb). Chloroplast autofluorescence is shown in red. Bars=20 μm.

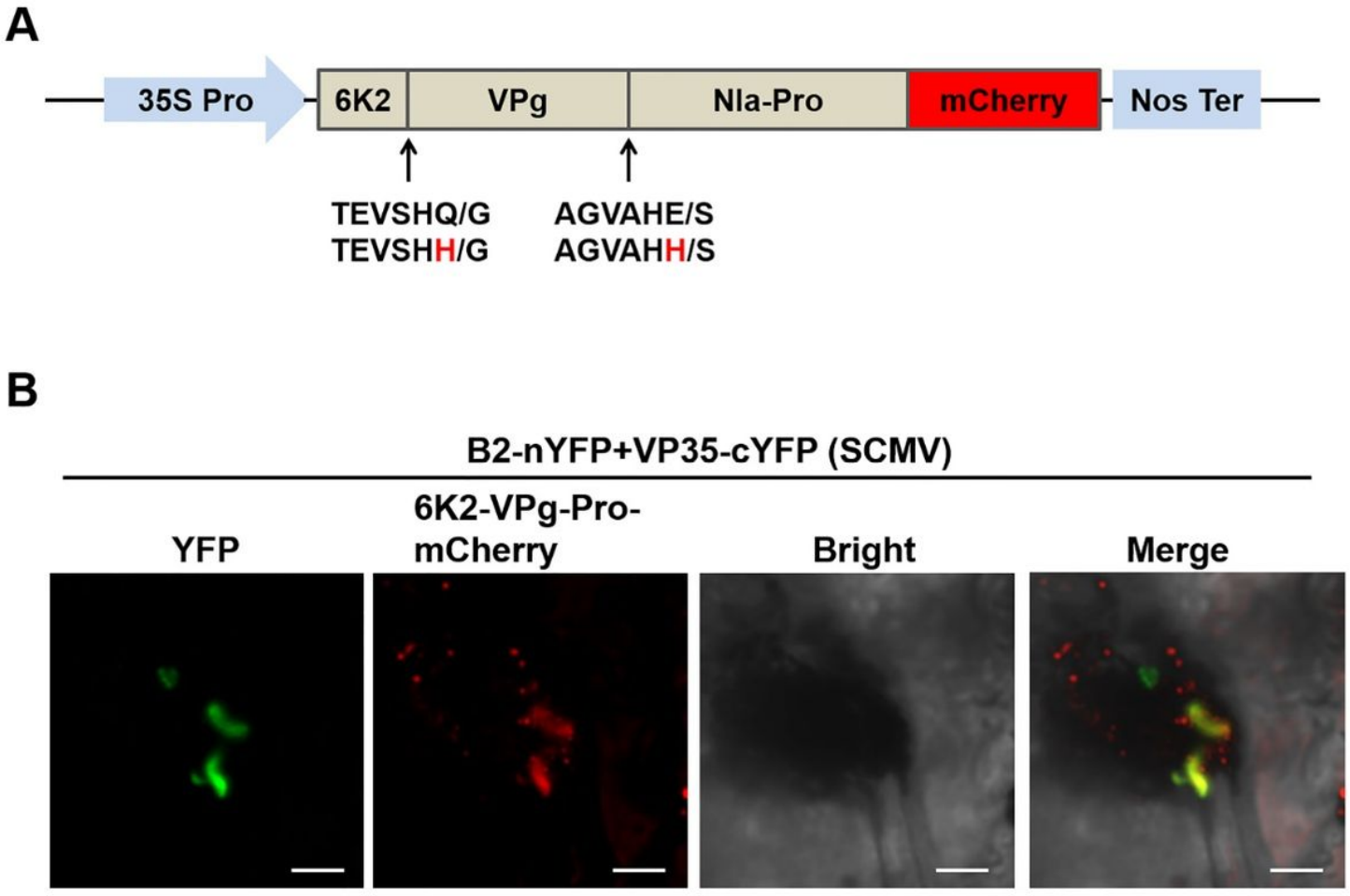

Figure 3

SCMV replicative dsRNA is associated with 6K2-VPg-Pro-induced vesicles. (A) Schematic representation of construct 6K2-VPg-Pro-mCherry. SCMV-encoded precursor 6K2-VPg-Pro polyprotein was fused with mCherry at C-terminus, and expressed under cauliflower mosaic virus 35S promoter (35S Pro). The cleavage sites and adjacent amino acid sequences in 6K2-VPg and VPg-Nla-Pro are shown under the arrows indicating junctions between 6K2 and VPg, VPg and Nla-Pro. Note that both Glutamine (Q) and Glutamic acid (E) residues are replaced by histidine (H) to prevent protein proteolysis. NOS Ter, NOS Terminator. (B) SCMV replication sites localize to 6K2-VPg-Pro-induced vesicles in maize leaf cells. 6K2-VPg-Pro-mCherry and split B2-nYFP and VP35-cYFP were co-expressed in SCMV-infected maize leaf cells by particle bombardment assay, followed by confocal microscopic analysis at 20 hpb. Bars=10 μm.

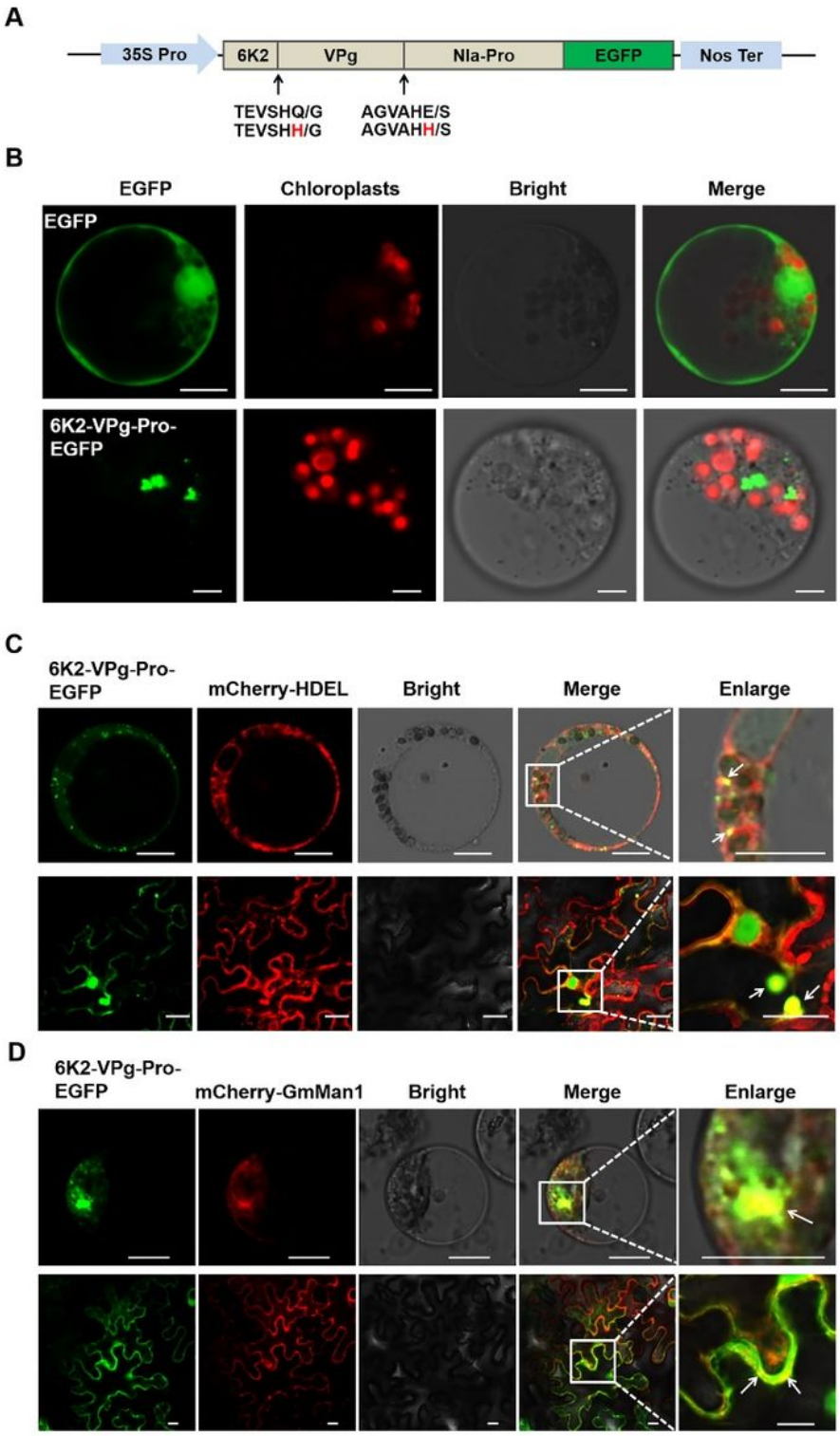

Figure 4

SCMV 6K2-VPg-Pro localizes to multiple subcellular organelles apart from chloroplasts. (A) Schematic showing the construct 6K2-VPg-Pro-EGFP. SCMV-encoded precursor 6K2-VPg-Pro polyprotein was fused with EGFP at C-terminus, and expressed under 35S Pro. Note that both Glutamine (Q) and Glutamic acid (E) residues are replaced by histidine (H) to prevent protein proteolysis. 35S Pro, 35S promoter; NOS Ter, NOS Terminator. (B) 6K2-VPg-Pro has no association with chloroplasts. Plasmids expressing EGFP or

6K2-VPg-Pro-EGFP were transfected into maize protoplasts, followed by confocal microscopic analysis at 20 hpt. Chloroplast autofluorescence was displayed in red. Bars=10 μm. (C) Colocalization of 6K2-VPg-Pro with ER. Confocal analysis of cells co-expressing 6K2-VPg-Pro-EGFP and mCherry-HDEL (ER marker) in maize protoplasts (upper panel) and N. benthamiana leaf epidermal cells (below panel) at 20 hpt and 72 hpi, respectively. White arrows indicate colocalization of 6K2-VPg-Pro-EGFP and mCherry-HDEL. Bars=10 μm. (D) Colocalization of 6K2-VPg-Pro with Golgi apparatus. Confocal images showing cells co-expressing 6K2-VPg-Pro-EGFP and mCherry-GmMan1 (Golgi marker) in maize protoplasts (upper panel) and N. benthamiana leaf epidermal cells (below panel) at 20 hpt and 72 hpi, respectively. White arrows indicate colocalization of 6K2-VPg-Pro-EGFP and Golgi marker. Bars=7.5 μm.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Supplementaryfile1.doc
- Supplementaryfile2.doc