Sugarcane mosaic virus remodels multiple intracellular organelles to form genomic RNA replication sites

Jipeng Xie1 · Tong Jiang1 · Zhifang Li1 · Xiangdong Li2 · Zaifeng Fan1 · Tao Zhou1

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Abstract
Positive-stranded RNA viruses usually remodel the host endomembrane system to form virus-induced intracellular vesicles for replication during infections. The genus Potyvirus of the family Potyviridae represents the largest number of positive single-stranded RNA viruses, and its members cause great damage to crop production worldwide. Although potyviruses have a wide host range, each potyvirus infects a relatively limited number of host species. Phylogenesis and host range analysis can divide potyviruses into monocot-infecting and dicot-infecting groups, suggesting that they differ in their infection mechanisms, probably during replication. Comprehensive studies on the model dicot-infecting turnip mosaic virus have shown that the 6K2-induced replication vesicles are derived from the endoplasmic reticulum (ER) and subsequently target chloroplasts for viral genome replication. However, the replication site of monocot-infecting potyviruses is unknown. In this study, we show that the precursor 6K2-VPg-Pro polyproteins of dicot-infecting potyviruses and monocot-infecting potyviruses cluster phylogenetically in two separate groups. With a typical gramineae-infecting potyvirus—sugarcane mosaic virus (SCMV)—we found that replicative double-stranded RNA (dsRNA) forms aggregates in the 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 the ER, Golgi apparatus, mitochondria, and peroxisomes, and have no evident association with chloroplasts.

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 that provide a 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 compartments are subverted and modified by different viruses infecting plants and animals [4–6]. In particular, the endoplasmic reticulum (ER) is targeted by various viruses to induce invagination of its outer membrane and form spherules or vesicles [7–13]. In addition to the ER, chloroplasts, mitochondria, peroxisomes, tonoplasts, the nuclear membrane, and the plasma membrane can also be rearranged to form membrane-bound VRCs in infected cells, indicating that these cellular membrane compartments are sites of replication of the corresponding viruses [14–19]. These membranous vesicles are thought to function in concentrating viral components and confine virus replication to a specific compartment to escape the host defense response and thereby to support the efficient replication of the viruses [6, 20].

The genus Potyvirus includes agriculturally and economically important viruses that cause great damage to 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 that is subsequently processed into 10 mature proteins [22–24]. In addition, a P3N-PIPO resulting from viral RNA polymerase slippage is expressed within the 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 turnip mosaic virus (TuMV) has been demonstrated to be responsible for the induction of ER-derived cytoplasmic vesicles containing VRCs consisting of RNA-dependent RNA polymerase (RdRp), 6K1, P3, C1, 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, as has been shown for viral RNAs of tobacco etch virus (TEV) and potato virus Y (PVY) [33, 34]. Further studies have provided clear evidence that 6K2-induced vesicles accumulate at the ER membrane, migrate to the Golgi apparatus, and then target the 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 dicot-infecting potyviruses [37, 38]. Considering this phylogenetic difference, the replication mechanisms of dicot- and monocot-infecting potyviruses may be distinct. There is considerable evidence that both the 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 staple food crops in the world [39, 40]. The potyvirus sugarcane mosaic virus (SCMV) is a prevalent viral pathogen that causes maize dwarf mosaic disease worldwide [41, 42]. In addition to maize, SCMV can infect several other 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 attaining a precise understanding of the virus replication process and its pathogenesis. In this study, we provide evidence 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 polyproteins were downloaded from the GenBank database at the National Center for Biotechnology Information and then subjected to multiple sequence alignment and phylogenetic analysis using MAFFT online [43]. A phylogenetic tree was constructed by the neighbor-joining method. The phylogeny was tested by 500 bootstrap resamplings, using the Poisson model as the amino acid substitutions type.

Plasmid construction

The coding regions of 6K2, Nla-VPg, and Nla-Pro were amplified by PCR using an SCMV-BJ infectious clone as the template [44] with primers that were designed to modify the cleavage sites at the junction of 6K2 and Nla-VPg and the junction of Nla-VPg and Nla-Pro to prevent polyprotein proteolysis (Supplementary Table 1) [45]. PCR products of 6K2, Nla-VPg, and Nla-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 the 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

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

Isolation and transfection of maize protoplasts

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 the dark at 24°C to obtain etiolated plants [49, 50]. Maize protoplasts were isolated and transfected as described previously [49, 51].

Particle bombardment assay

For the particle bombardment assay, mock-inoculated or SCMV-BJ-infected maize plants that were sap-inoculated at the 3-leaf-stage were allowed to grow to the 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 of plasmids, 20 μL of 0.1 M spermidine, and 50 μL of 2.5 M CaCl₂. After vortexing for 3 min, the resulting mixture was centrifuged at 10000 g for 20 s. The pellet was resuspended and washed twice with 140 μL of 70% ethanol. Finally, the pellet was resuspended in 10 μL of absolute ethanol for bombardment. The bombardment assay was performed using a PDS-1000/He system (Bio-Rad) as instructed. The helium
pressure was regulated at 1,500 pounds per square inch. The bombardered maize leaf tissues were kept in the dark at 25°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 the freeze-thaw method [53]. The bacteria were then cultured overnight, centrifuged to obtain a pellet, and resuspended in MMA buffer containing 10 mM MgCl₂, 10 mM MES, pH 5.6, and 100 mM acetosyringone. The resuspended bacteria were then diluted to an OD₆₀₀ of 0.5, followed by incubation at room temperature for 2-3 h and 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 Leica TCS SP8 confocal laser-scanning microscope. The fluorescence of both EGFP and YFP was excited at 488 nm, and mCherry and chloroplast autofluorescence was excited at 552 and 638 nm, respectively. The "between-lines" sequential scanning mode was used to avoid overlapping fluorescence from different fluorophores.

**Western blot analysis**

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

**Results**

**6K2-VPg-Pro polyproteins of monocot-infecting potyviruses are phylogenetically separate from those of dicot-infecting potyviruses**

To investigate whether monocot-infecting and dicot-infecting potyviruses use the same replication site, we made 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 in MAFFT online [43]. The multiple sequence alignment showed that the 6K2-VPg-Pro polyproteins of 20 representative potyviruses shared 58.55% sequence identity and that 54 residues were conserved in all of the sequences (Supplementary Fig. 1). Strikingly, the residues 39W, 299N, 413S were relatively conserved in dicot-infecting potyviruses, whereas these three sites in monocot-infecting potyviruses were mostly conserved as 39L, 299P, and 413D (Supplementary Fig. a). The phylogenetic tree showed two main clades, including a monocot-infecting cluster and a dicot-infecting potyviruses cluster (Fig. 1). 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 consisted only of dicot-infecting potyviruses (Fig. 1).

**SCMV replication sites do not correspond to 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, are formed during virus replication, and the site where they are found is presumed to be the site of virus replication. To label the sites of SCMV replication in maize cells, we performed a dsRNA binding-dependent fluorescence complementation (dRBFC) assay by co-expressing B2-nYFP and VP35-cYFP in SCMV-infected maize cells as described previously [36]. In this assay, maize protoplasts isolated from mock-infected or systemically SCMV infected leaves (see Materials and 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 the cytoplasm of SCMV-infected maize protoplasts (Fig. 2A), and the reconstituted YFP signals had no obvious association with chloroplast...
autofluorescence (Fig. 2A). No fluorescent signal was observed in mock-infected maize protoplasts (Fig. 2A). Western blotting confirmed the infection of maize protoplasts by SCMV (Supplementary Fig. 2A).

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

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

Studies on dicot-infecting potyviruses such as TEV and TuMV have 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 investigate whether SCMV-encoded 6K2-VPg-Pro can induce cytoplasmic vesicle structures that 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 its C-terminus (Fig. 3A). It should be noted that the glutamine (Q) and glutamic acid (E) residues preceding the cleavage sites at the 6K2-VPg and VPg-NIa-Pro junctions were both replaced 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 the granular YFP foci formed by dsRNA binding of B2-nYFP and VP35-cYFP overlapped perfectly 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.
RNA replication sites of sugarcane mosaic virus

Fig. 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. A YFP signal was observed in SCMV-infected maize protoplasts at 20 hours post-transfection (hpt) by confocal microscopy. Chloroplast autofluorescence is shown 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.

Fig. 3 SCMV replicative dsRNA is associated with 6K2-VPg-Pro-induced vesicles. (A) Schematic representation of the construct 6K2-VPg-Pro-mCherry. The SCMV-encoded precursor 6K2-VPg-Pro polyprotein was fused with mCherry at the C-terminus and expressed under a 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 the junctions between 6K2 and VPg and between VPg and Nla-Pro. Note that both glutamine (Q) and glutamic acid (E) residues were 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.
6K2-VPg-Pro-induced replication vesicles reside on multiple intracellular organelles, excluding 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. To test this, we made the construct 6K2-VPg-Pro-EGFP by fusing EGFP to the C-terminus of 6K2-VPg-Pro (Fig. 4A) and delivered it into protoplasts isolated from systemically SCMV-infected maize leaves. In contrast to unfused EGFP, which localized in the nucleus and cytoplasm, aggregate structures showing green fluorescence could only be observed in the cytoplasm of protoplasts transfected with 6K2-VPg-Pro-EGFP (Fig. 4B). Unexpectedly, these structures clearly had no obvious association with the red autofluorescence of chloroplasts (Fig. 4B). Moreover, in N. benthamiana epidermal cells, transient expression of either 6K2-VPg-Pro-EGFP or 6K2-VPg-Pro-mCherry also resulted in the formation of aggregate bodies and punctate structures in the cytoplasm (Supplementary Fig. 3). The fluorescence from these aggregate bodies and punctate structures did not merge with the chloroplast autofluorescence (Supplementary Fig. 3), suggesting that SCMV 6K2-VPg-Pro-induced replication vesicles do not reside on chloroplasts.

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

We further attempted 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 whose fluorescence merged with that of mCherry-ScCOX4 [57] and the peroxisome marker dsRed-SKL [58] in N. benthamiana leaf epidermal cells (Supplementary Fig. 4). Taken together, these results show that SCMV can target multiple intracellular organelles for replication, including the ER, Golgi apparatus, mitochondria, and peroxisomes, but not chloroplasts.

Discussion

In this study, we investigated the location of active replication sites of SCMV in maize cells using a 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, and barley stripe mosaic virus [36, 59]. Given that the dRBFC assay requires active viral replication, which generates double-stranded replicative RNA intermediates [36], we used young maize leaves already systemically infected with SCMV for protoplast preparation and bombardment to locate the replication sites of SCMV. Systemic infection of young leaves with SCMV ensures that the virus is in an active replication state in the maize cells. The dRBFC assay clearly showed that SCMV replicative dsRNA localized in the cytoplasm but did not reside on chloroplasts in maize protoplasts or 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 the observation 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 those induced by TuMV 6K2 [35]. The punctate or aggregated appearance may result from the membrane-binding and fusion properties of these vesicles [8]. Some of the SCMV 6K2-VPg-Pro-induced vesicles colocalized perfectly with dsRNA replicative intermediates, indicating virus replication, while some were scattered in the
cytoplasm rather than colocalizing with dsRNA (Fig. 3B). Since the 6K2-Vpg-Pro-induced vesicles colocalized with dsRNA binding proteins (B2-YN and VP35-YC), it is likely that the SCMV 6K2-Vpg-Pro-induced vesicles provide compartments for active SCMV RNA replication.

Studies on TuMV 6K2 have revealed that it can induce small mobile vesicles derived from the ER at an early stage of infection and form an irregularly shaped structure juxtaposed to the nucleus later in infection [8]. TuMV RNA replication takes place within these 6K2 vesicles, associating taposed to the nucleus later in infection [8]. TuMV RNA of infection and form an irregularly shaped structure jux-

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