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Rice Stripe Tenuivirus NSvc2 Glycoproteins Targeted to Golgi Body by N-Terminal Transmembrane Domain and Adjacent Cytosolic 24 Amino-Acids via COP I- and COP II-Dependent Secretion Pathway

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Abstract

The NSvc2 glycoproteins encoded by *Rice stripe tenuivirus* (RSV) share many characteristics common to the glycoproteins found among *Bunyaviridae*. Within this viral family, glycoproteins targeting to the Golgi apparatus play a pivotal role in the maturation of the enveloped spherical particles. RSV particles, however, adopt a long filamentous morphology. Recently, RSV NSvc2 glycoproteins were shown to localize exclusively to the ER in Sf9 insect cells. Here, we demonstrate that the amino-terminal NSvc2 (NSvc2-N) targets to the Golgi apparatus in *Nicotiana benthamiana* cells, whereas the carboxyl-terminal NSvc2 (NSvc2-C) accumulates in the ER. Upon co-expression, NSvc2-N redirects NSvc2-C from the ER to the Golgi. The NSvc2 glycoproteins move together with the Golgi stacks along the ER/actin network. The targeting of the NSvc2 glycoproteins to the Golgi was strictly dependent on functional anterograde traffic out of the ER to the Golgi or on a retrograde transport route from the Golgi apparatus. The analysis of truncated and chimeric NSvc2 proteins demonstrates that the Golgi targeting signal comprises amino acids 269-315 of NSvc2-N, encompassing the transmembrane domain and 24 adjacent amino acids in the cytosolic tail. Our findings demonstrate for the first time that the glycoproteins from an unenveloped *Tenuivirus* could target into Golgi bodies in plant cells.

Importance

NSvc2 glycoprotein encoded by unenveloped *Rice stripe tenuivirus* (RSV) share many characteristics in common with glycoprotein found among *Bunyaviridae* in which all members have membrane-enveloped sphere particle. Recently, RSV NSvc2 glycoproteins were shown to localize exclusively to the ER in Sf9 insect cells. In this study, we demonstrated that the RSV glycoproteins could target into Golgi in plant cells. The targeting of NSvc2 glycoproteins to the Golgi was dependent on active COP II or COP I. The Golgi targeting signal was mapped to the 23-amino-acids transmembrane domain and the adjacent 24-amino-acids of the cytosolic tail of the NSvc2-N. In light of the evidence from viruses in Bunyavidae that targeting into Golgi is important for the viral particle assembly and vector transmission, we propose that targeting of RSV glycoproteins into Golgi in plant cells represents a physiologically relevant mechanism in the maturation of RSV particle complex for insect vector transmission.

INTRODUCTION

Rice stripe virus (RSV) is the type member of the genus *Tenuivirus* (1). RSV has caused severe damage to rice crops in China and is known to be transmitted by *Laodelphax striatellus* in a persistent, circulative-propagative manner (2). The RSV genome consists of four negative-sense single-stranded RNA segments, designated RNA1, 2, 3 and 4, which encode seven ORFs using a negative or ambisense coding strategy (3). RNA1 is negative sense and encodes an RNA-dependent RNA polymerase (RdRp) (4). The other three segments adopt an ambisense coding strategy. RNA2 encodes a 22.8 kDa protein (NSs2) from the viral RNA (vRNA) and a 94 kDa protein (NSvc2) from the viral complementary RNA (vcRNA) (5). RNA3 encodes a viral suppressor (NSs3, 23.9 kDa) from the vRNA (6) and a nucleocapsid protein (NSvc3, 35 kDa) from the vcRNA (7, 8). RNA4 encodes a 20.5 kDa protein (NSs4) from the vRNA and a movement protein (NSvc4, 32 kDa) from the vcRNA (9).

Based on phylogenetic relationship and their genome organization and gene expression strategies, tenuiviruses are more closely related to the animal-infecting viruses in the genus *Phlebovirus* of the family *Bunyaviridae* than they are to plant tospoviruses (10). The NSvc2 protein encoded by RSV (hereinafter the NSvc2 glycoprotein) shares many characteristics in common with the glycoproteins found in the *Bunyaviridae* family of viruses in which all members adopt an enveloped spherical virion form (10). The glycoprotein encoded by the *Bunyaviridae* viruses is processed into two proteins, Gn (the amino-terminal glycoprotein) and Gc (the

carboxyl-terminal glycoprotein), which together form the surface spikes of the mature enveloped virion (11-14). The Gn protein of several viruses, including *Uukuniemi virus* (UUKV) (15), the *Punta toroviruses* (16), and *Rift valley fever virus* (RVFV) (17) in the genus *Phlebovirus,* as well as *Tomato spotted wilt tospovirus* (TSWV) (18), has been shown to accumulate in the Golgi apparatus, while the Gc protein localizes to the endoplasmic reticulum (ER). Upon co-expression, both glycoproteins localize to the Golgi apparatus (16-19), suggesting that Gn can re-target Gc from the ER to the Golgi. The targeting of the viral glycoproteins to the Golgi apparatus plays a pivotal role in the maturation of the viral particles. The NSvc2 glycoprotein encoded by RSV was predicted to be functionally similar to the glycoproteins found on other *Bunyaviridae* viruses. RSV particles, however, adopt a long filamentous morphology unenveloped (19, 20). The enveloped nature of *Bunyaviridae* versus the unenveloped nature of *Tenuivirus* raises the question of what common or unique strategies have evolved for them to form different morphology of viral particle. Zhao *et al.* (2012) recently reported that the NSvc2 protein, or its two processing products, the amino-terminus of NSvc2 (NSvc2-N) and the carboxyl-terminus of NSvc2 (NSvc2-C), exclusively localized to the ER membrane in *Spodoptera frugiperda* (Sf9) insect cells (21). It remains poorly understood whether the ER localization (the inability to target to the Golgi apparatus) of the NSvc2 glycoproteins is the key step determining the adoption of a long filamentous particle in RSV. It is also unknown why does a nonenveloped teniuvirus encode glycoproteins.

RSV systemically infects *Nicotiana benthamiana* by mechanical inoculation (9, 22). In this study, the subcellular targeting of the NSvc2 glycoproteins and the requirements for their targeting were extensively characterized in *N. benthamiana*. We demonstrated that the NSvc2-N glycoprotein alone is able to target to the Golgi apparatus in *N. benthamiana*, whereas NSvc2-C localizes to the ER membrane in the absence of NSvc2-N. Upon co-expression, NSvc2-N redirects NSvc2-C to the Golgi apparatus. The NSvc2 glycoproteins were found to move together with the Golgi stacks along the ER/actin network in *N. benthamiana* epidermal cells. Using dominant-negative mutants, we demonstrated that the targeting of the NSvc2 proteins from the ER to the Golgi was strictly dependent on COP I and COP II early secretion pathways. The analysis of truncated and chimeric NSvc2 proteins demonstrated that the Golgi targeting signal localized to amino acids 269-315, encompassing the 23-amino acid transmembrane domain and the 24 adjacent amino acids of the cytosolic tail. Our findings provide novel insights into the cellular properties of RSV glycoproteins in plant cells.

MATERIALS AND METHODS

Plasmid constructs and organelle markers

p1300S-NSvc2-N-YFP and p1300S-NSvc2-C-YFP. NSvc2-N and NSvc2-C were amplified from total RNA isolated from rice infected by RSV using RT-PCR and the primers XT746/XT747 and XT800/XT388 (Supplemental Table S1). The NSvc2-N and NSvc2-C PCR fragments were digested with *Kpn* I and *Bam*H I and inserted into

p1300S-YFP using the same restriction sites to obtain p1300S-NSvc2-N-YFP and p1300S-NSvc2-C-YFP, respectively.

p1300S-NSvc2-Intron-YFP. A potato ST-LS1 intron (23) was inserted into the AG/GT site at nucleotide (nt) position 1182 of NSvc2. The ST-LS1 intron, N-terminal fragment (1182 nt) and C-terminal fragment (1423 nt) of NSvc2 were amplified using the primers XT957/XT958, XT746/XT959 and XT960/XT388, respectively. The three PCR fragments were mixed and amplified using XT746/XT388 to obtain NSvc2-Intron, which was then digested with *Kpn* I and *Bam*H I and inserted into p1300S-YFP using the same restriction sites.

p1300S-NSvc2-N-46del-YFP and p1300S-NSvc2-N-63del-YFP. NSvc2-N containing either a 46 or 63 amino acid deletion at the C-terminus was amplified using the primer pairs XT746/XT807 or XT746/XT835, and the PCR products were inserted into the *Kpn* I and *Bam*H I sites of p1300S-YFP, respectively.

p1300S-$SS_N$$TMD_N$$CT_N$-YFP, p1300S-$SS_N$$TMD_N$$CT_N$del46-YFP and p1300S-$SS_N$$TMD_N$$CT_N$del63-YFP. The signal peptide (SS_N), transmembrane domain (TMD_N) containing the full-length cytosolic domain (CT_N), TMD_N containing the CT_N with a 46 amino acid deletion and the TMD_N with the CT_N containing a 63 amino acid deletion at the C-terminus of NSvc2-N were amplified using the corresponding primer pairs (XT746/XT837, XT836/XT747, XT836/XT807 and

XT836/XT835). The $SS_NTMD_NCT_N$, $SS_NTMD_NCT_N$del46, and $SS_NTMD_NCT_N$del63 fragments were fused using overlap PCR and the primers XT746/XT747, XT746/XT807 and XT746/XT835, and were inserted into the *Kpn* I and *Bam*H I sites of p1300S-YFP, respectively.

p1300S-NSvc-C(TMD_NCT_N)-YFP and p1300S-NSvc-C(TMD-CT-del46)-YFP. A fragment of NSvc2-C lacking the TMD_C and the CT_C was amplified using the primers XT800 and XT869. The TMD_N fragment with the full-length CT_N and the TMD_N fragment with the CT_N containing a 46 amino acid deletion at the C-terminus of NSvc2-N were amplified with the primer pairs XT747/XT868 and XT807/XT868. They were then fused using overlap PCR and the primers XT800/XT747 and XT800/XT807, respectively. The products of overlap PCR were digested with *Kpn* I and *Bam*H I and cloned into p1300S-YFP.

p1300S-CFP-Sec24 and p1300S-Arf1-CFP. The full-length Sec24 (AT3G07100) and Arf1 genes were amplified using RT-PCR and the total RNA extracted from the Col ecotype of *Arabidopsis thaliana* using the primers XT743/XT754 and XT784/XT785, respectively. The Sec24 PCR fragments were digested with *Bam*H I and cloned into the *Bgl* II site of p1300S-CFP, while Arf1 was digested with *Bam*H I and cloned into the *Bam*H I site of p1300S-CFP.

p1300S-Arf1 (T31N). To construct p1300S-ArfI (T31N), site-directed mutagenesis was used to introduce the mutation into Arf1 using the primers XT784/XT795 and XT794/XT785 and overlap PCR. The PCR product was digested with *Bam*H I and cloned into p1300S.

The ER marker mCherry-HDEL (24) and the Golgi marker Man49-mCherry (24) were obtained from the Arabidopsis Biological Resource Center (ABRC). The Sar1 dominant-negative mutant construct Sar1 (H74L) was kindly provided by Professor Taiyun Wei (25).

Plant material, transient expression and treatment

RSV (Jiangsu isolate) was collected from infected rice in a field in Nanjing and frozen at -80°C until use. All transient expression experiments were performed using six- to eight-week old *N. benthamiana* plants. *Agrobacterium tumefaciens* cells (C58C1 containing various RSV constructs and organelle markers) were grown using kanamycin selection. The *Agrobacterium* cells were treated with infiltration buffer (10 mM $MgCl_2$, 10 mM MES, pH 5.9, and 150 μM acetosyringone) for 3 hr at room temperature before being infiltrated (OD600 = 0.5) into the abaxial surface of *N. benthamiana* leaves. All agroinfiltrated plants were grown in growth chambers (Model GXZ500D, Jiangnan Motor Factory, Ningbo, P. R. China) under a 16 h light/8 h dark cycle and a constant temperature of 25°C. The agroinfiltrated leaves were examined for fluorescence expression between 24-72 hpi. When applicable, LatB

(Sigma) was infiltrated at a final concentration of 10 μM into *N. benthamiana* leaves before fluorescence observation.

Confocal laser scanning microscopy

Leaf discs were dissected from the agroinfiltrated leaf area of *N. benthamiana* leaves and mounted in water between two cover slips. Images and movies were captured using a Carl Zeiss LSM 710 confocal laser scanning microscope and 20×, 63× oil or 63× water immersion objective lenses. CFP fluorescence was excited at 405 nm and emission captured at 440-470 nm, YFP were excited at 488 nm and emission captured at 497-520 nm, and mCherry was excited at 561 nm and emission captured at 585-615 nm. Images were processed using the Zeiss 710 CLSM and Adobe Photoshop programs (San Jose, CA, USA). Movies were edited using the Corel Video Studio Pro X4 software (Ottawa, Ontario, Canada).

Western blot analysis

Plant leaves from *N. benthamiana* agroinfiltrated with NSvc2-N-YFP, NSvc2-C-YFP and NSvc2-YFP constructs were ground in a 1:3 (w/v; 0.1 g/300 μL) ratio of extraction buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 10% glycerol, 0.1% Triton X-100 and 1×plant protease inhibitor). After centrifugation for 10 min at 3,000 × *g*, the supernatant of the total protein preparation was separated by SDS-polyacrylamide gel electrophoresis for immunoblot analysis. The blots were probed with anti-YFP (Polyclonal antibody, 1:1,000 dilution; Biyuntian, Shanghai,

China) and visualized with AP conjugated Goat anti-rabbit secondary antibodies (1:1,000 dilution; Biyuntian, Shanghai, China) followed by nitro-blue tetrazolium (NBT) and 5-bromo-4-chloro-3'-indolyphosphate (BCIP) staining (ready-made solutions; Shenggong, Shanghai, China).

For subcellular fractionations, the soluble and microsomal fractions were isolated from *N. benthamiana* leaves agroinfiltrated with NSvc2-N-YFP, NSvc2-C-YFP and NSvc2-YFP constructs as described by Peremyslov *et al.* (2004) (26). The antigens on the membranes were blotted with anti-YFP (rabbit). It was detected by DyLight 680-coupled goat anti-rabbit antibodies (1:10,000 dilution; Pierce, IL USA) and then visualized by Licor Odyssey scanner.

RESULTS

The NSvc2-N protein is targeted to the Golgi apparatus

N. benthamiana is an ideal plant species in which to assess the subcellular localization of viral proteins. To characterize the subcellular target of the NSvc2 glycoproteins in plant cells, we first fused the yellow fluorescent protein (YFP) to the C-terminus of NSvc2-N (Fig. 1) and then agroinfiltrated the construct into *N. benthamiana* epidermal cells. Western blot analysis showed that NSvc2-N-YFP fusion protein was expressed as a size of 68kDa protein (Fig. 2A), indicating a proper expression of the NSvc2-N-YFP construct. To investigate the intracellular localization

of the NSvc2-N-YFP protein, we isolated soluble (S30) and microsomal (P30) protein fractions from *N. benthamiana* leaves agroinfiltrated with NSvc2-N-YFP. We found that NSvc2-N-YFP was localized exclusively in microsomal fractions that are known to contain ER membrane structures and Golgi bodies (Fig. 2B).

To further characterize the subcellular localization of NSvc2-N-YFP, the infiltrated leaves were examined using Zeiss 710 confocal laser scanning microscopy. At 36 hours post-infiltration (hpi), NSvc2-N-YFP was observed as numerous small bodies in the cortical cytoplasm of the cells (Fig. 2C). To determine whether NSvc2-N accumulated in the ER membrane, we co-expressed the NSvc2-N-YFP protein with the HDEL signal fused to the N-terminus of mCherry (mCherry-HDEL) in *N. benthamiana* (24). The merge of NSvc2-N-YFP with mCherry-HDEL images revealed that the NSvc2-N-YFP signal did not colocalize with the ER marker, while those NSvc2-N-YFP punctate bodies were still associated with the ER membrane (Fig. 2C-E).

To determine whether the NSvc2-N-YFP bodies co-localized with the Golgi stacks, we co-infiltrated the Golgi marker construct Man49-mCherry (24) with NSvc2-N-YFP in *N. benthamiana* epidermal cells. At 36 hpi, we found that the NSvc2-N-YFP bodies co-localized with the Golgi stacks (Fig. 2F-H), suggesting that the NSvc2-N-YFP protein targets to the Golgi apparatus. We then examined the NSvc2-N-YFP protein signal at three time points, 24, 48 and 72 hpi, and found that

NSvc2-N-YFP was targeted to the Golgi body as early as 24 hpi.

The NSvc2-C protein accumulates in the ER membrane

We also fused NSvc2-C protein with YFP at its C-terminus (Fig. 1) and infiltrated the construct into *N. benthamiana* epidermal cells. Immunoblot analysis showed that NSvc2-C-YFP protein expressed as 78kDa protein which is same as the predicted size of NSvc2-C-YFP fusion protein (Fig. 3A). Fractionation analysis revealed that NSvc2-C-YFP protein was localized only in the microsomal membrane fractions (Fig. 3B). To precisely define the intracellular distribution of NSvc2-C, the infiltrated leaves were characterized using confocal laser scanning microscopy. The green fluorescent signal of the NSvc2-C-YFP fusion protein appeared to be very weak, but was still detectable in an ER-like network structure observed at 36 hpi (Fig. 3C). To determine whether these fluorescent signals co-localized with the ER structure, the cortical ER marker mCherry-HDEL was co-infiltrated with NSvc2-C-YFP. As shown in Fig. 3C-E, the NSvc2-C-YFP protein co-localized with the ER membrane network.

To examine whether NSvc2-C-YFP accumulated in the Golgi stacks, we co-infiltrated *N. benthamiana* cells with NSvc2-C-YFP and the Golgi marker Man49-mCherry. As shown in Fig. 3F-H, no fluorescent signal associated with NSvc2-C-YFP was found to accumulate in the Golgi apparatus. To confirm whether NSvc2-C-YFP exhibits any accumulation in the Golgi stacks, we checked the fluorescent signal of NSvc2-C-YFP

at 24, 48 and 72 hpi. The NSvc2-C-YFP protein did not form any small bodies that could target to the Golgi body at the three time points examined. These results suggest that NSvc2-C-YFP was arrested in the ER in *N. benthamiana*.

The NSvc2-N protein recruits NSvc2-C from the ER to the Golgi apparatus

To determine the localization and trafficking of the NSvc2 glycoproteins when expressed from their precursor, we fused YFP to the C-terminus of the NSvc2 precursor protein. However, the construct containing the full-length NSvc2 gene cannot grow in *E. coli* cells, suggesting that the full-length NSvc2 gene is toxic to *E. coli*. We therefore inserted a potato ST-LS1 intron (23) into the AG/GT site at nucleotide (nt) position 1182 of NSvc2. The intron-containing construct, NSvc2-Intron-YFP (Fig. 1), can successfully generate a green fluorescence signal in *N. benthamiana* epidermal cells after agroinfiltration. Total RNA was then isolated from infiltrated leaves and the NSvc2-Intron-YFP RT-PCR products were sequenced to confirm that the intron had been precisely processed from the inserted site of NSvc2 (NSvc2-Intron-YFP is hereinafter referred to as NSvc2-YFP). Immunoblot analysis showed that NSvc2-C-YFP has been efficiently processed from precursor protein NSvc2-YFP and expressed as 78 kDa protein (Fig. 4A). The processed protein was distributed exclusively in the microsomal fractions which are known to contain ER membranes and Golgi bodies (Fig. 4B).

We then co-expressed NSvc2-YFP with the ER marker mCherry-HDEL in *N. benthamiana* and the infiltrated leaves were examined using Zeiss confocal laser scanning microscopy. Monitoring of NSvc2-C-YFP (NSvc2-C-YFP processed from the NSvc2 precursor) showed that the fluorescent signal highlighted by NSvc2-C-YFP co-localized in the ER network at 24-48 hpi. At 48-72 hpi, NSvc2-C-YFP began to induce punctate structures along the ER membrane in the presence of NSvc2-N (Fig. 4C-E). To identify whether the newly formed bodies targeted to the Golgi apparatus, we co-infiltrated *N. benthamiana* with NSvc2-YFP and the Golgi marker Man49-mCherry. As shown in Fig. 4F-H, NSvc2-C-YFP bodies were indeed found to be targeted to the Golgi apparatus. These results strongly suggest that NSvc2-N is able to recruit NSvc2-C from the ER to the Golgi apparatus.

Targeted NSvc2 glycoproteins move together with the Golgi stacks in *N. benthamiana*

In tobacco leaf cells, Golgi bodies traffic on an underlying ER track in an actin-dependent manner (27, 28). To examine whether the targeted RSV NSvc2 glycoproteins move with the Golgi bodies, we utilized time-lapse confocal microscopy to monitor the movement of NSvc2-N-YFP or NSvc2-N/NSvc2-C-YFP (processed from the NSvc2-YFP precursor) in the presence of the Golgi marker. Fig. 5A-C and D-F show examples of the movement of the NSvc2-N-YFP and NSvc2-N/NSvc2-C-YFP bodies with the Golgi stacks, and the arrows mark the progressive movement of these bodies in each sequence. We found that both

NSvc2-N-YFP and NSvc2-N/NSvc2-C-YFP moved together with the Golgi bodies (Fig. 5A-C and D-F; Supplemental Video S1 and S2).

To determine whether the movement of bodies labeled with NSvc2-N-YFP or NSvc2-N/NSvc2-C-YFP is dependent on similar forces driving the movement of the Golgi bodies, we treated agroinfiltrated leaves at 48 hpi with 10 μM latrunculin B, an actin depolymerizing agent (29). After 3 h of chemical treatment, we found that movement of the NSvc2-N-YFP or NSvc2-N/NSvc2-C-YFP as well as Golgi bodies was completely inhibited. However, NSvc2-N-YFP, NSvc2-N/NSvc2-C-YFP and the Golgi bodies remained co-localized (Supplemental Video S3 and S4). These data suggest that the NSvc2-N-YFP or NSvc2-N/NSvc2-C-YFP bodies move together with the Golgi stacks along the ER/actin network.

ER-to-Golgi targeting of NSvc2 glycoproteins is dependent on a functional COP II complex

Given that the RSV NSvc2-N-YFP and NSvc2-YFP fusion proteins targeted to the Golgi, we ask whether the Golgi targeting of viral glycoproteins results from traffic out of the ER to the Golgi apparatus via ERES. To address this question, we co-infiltrated an ERES-marker, CFP-Sec24 (30), with NSvc2-N-YFP or NSvc2-YFP proteins into *N. benthamiana* leaf cells. As shown in Fig. 6A-C and G-I, the NSvc2-N-YFP or NSvc2-YFP bodies co-localized with CFP-Sec24 fluorescence at the ERES. These results suggest that NSvc2-N is able to redirect NSvc2-C from the

ER to the ERES, from where they subsequently co-migrate, most likely as a heterodimer, to the Golgi apparatus.

The COP II complex is responsible for anterograde traffic out of the ER to the Golgi apparatus (31). To test whether COPII vesicles are involved in ER-to-Golgi transport of RSV NSvc2 glycoproteins, wild-type Sar1 or its dominant-negative mutant (H74L) (32) was co-infiltrated with NSvc2-N-YFP or NSvc2-YFP together with the Golgi marker Man49-mCherry into *N. benthamiana.* As shown in Fig. 6D-F and J-L, upon co-expression of NSvc2-N-YFP or NSvc2-YFP with Sar1 (H74L), the florescence of NSvc2-N-YFP or NSvc2-YFP, as well as of the Golgi bodies, was retrieved back to the ER network, while co-expression with wild-type Sar1 did not cause the NSvc2-N-YFP or NSvc2-YFP bodies to redistribute back to the ER (data not shown). These results suggest that the accumulation of the RSV glycoproteins at the ERES and in the Golgi bodies is dependent on a functional anterograde secretion pathway.

The accumulation of the NSvc2 glycoproteins at the Golgi bodies depends on active COP I

To investigate whether the Golgi targeting of viral glycoproteins also involves retrograde traffic, we co-infiltrated Arf1 tagged with CFP, a COP I vesicle marker (33), with NSvc2-N-YFP or NSvc2-YFP in *N. benthamiana*. As shown in Fig. 7A-C and G-I, the NSvc2-N-YFP or NSvc2-YFP bodies co-localized with COP I vesicles labeled by Arf1-CFP.

To determine the dependency of the ER-to-Golgi transport of RSV NSvc2 glycoproteins on active COP I, wild-type Arf1 or Arf1 (T31N), a dominant-negative mutant of COP I (33, 34), was co-infiltrated with NSvc2-N-YFP or NSvc2-YFP along with the Golgi marker Man49-mCherry into *N. benthamiana*. We found that NSvc2-N-YFP or NSvc2-YFP as well as Man49-mCherry labeled Golgi bodies redistributed back to the ER membrane in the presence of the dominant-negative Arf1 (T31N) (Fig. 7D-E and J-L). However, the co-expression of wild-type Arf1 has no such effect (data not shown). These data demonstrate that the Golgi targeting of RSV glycoproteins is also dependent on an active retrograde export route.

The Golgi targeting signal resides in a region of NSvc2-N encompassing a transmembrane domain and the 24 adjacent amino acids of the cytosolic tail

Both the NSvc2-N-YFP and NSvc2-YFP expressed in *N. benthamiana* localized to the Golgi complex, indicating that the Golgi retention signal resides in the N-terminus of the NSvc2 protein. To map the domain responsible for the Golgi targeting of RSV NSvc2-N, a truncated NSvc2-N del46-YFP protein, where 46 amino acids at the C-terminal end of NSvc2-N within the cytosolic tail were deleted and fused with YFP (Fig. 1), was constructed and transiently expressed in *N. benthamiana*. The intracellular localization of this protein was determined by confocal fluorescence analysis after 48 hpi. As illustrated in Fig. 8A-C, the truncated NSvc2-N del46-YFP

protein was still capable of targeting to the Golgi complex. Subsequently, 63 amino acids of the C-terminal end of the NSvc2-N protein within the cytosolic tail were deleted (Fig. 1). This truncated NSvc2-N del63-YFP protein was no longer targeted to the Golgi apparatus (Fig. 8D-F), suggesting that the amino acids in the cytosolic tail are required for entering into the Golgi.

To determine the minimum region required for Golgi targeting, the predicted transmembrane domain (amino acids 269-291) and the entire cytosolic domain (amino acids 292-361) of NSvc2-N were fused with its signal peptide sequence (amino acids 1-23) (Fig. 1). When this chimeric $SS_NTMD_NCT_N$-YFP construct was expressed in *N. benthamiana* leaf cells, we found that it accumulated in the Golgi apparatus (Fig. 8G-I). Subsequently, the transmembrane domain and the 24 adjacent amino acids (CTdel46, amino acids 292-315) were fused with its signal peptide (Fig. 1). The resulting $SS_NTMD_NCT_N$del46-YFP construct also localized to the Golgi apparatus (Fig. 8J-L). Lastly, the transmembrane domain and the 7 adjacent amino acids (CTdel63, amino acids 292-298) were fused with its signal peptide (Fig. 1). As shown in Fig. 8M-O, this $SS_NTMD_NCT_N$del63-YFP construct was incapable of targeting to the Golgi complex. These analyses suggest that both the transmembrane domain (amino acids 269-291) and the 24 adjacent amino acids in the cytosolic tail of the NSvc2-N protein are required for Golgi targeting.

To substantiate the observation that the Golgi retention signal is located within the TMD and CT domains of NSvc2-N, the transmembrane domain (amino acids 269-291) and the entire cytosolic domain (amino acids 292-361) of NSvc2-N were swapped with those of NSvc2-C (Fig. 1). The resulting NSvc2-C(TMD_NCT_N)-YFP construct was co-expressed with mCherry-HDEL and Man49-mCherry separately in *N. benthamiana*. As shown in Fig. 8P-R, the chimeric NSvc2-C(TMD_NCT_N)-YFP construct was capable of targeting to the Golgi apparatus, suggesting that the transmembrane domain and the cytosolic domain of NSvc2-N was sufficient to direct NSvc2-C-YFP to the Golgi complex (Fig. 8P-R). To analyze the requirement for the Golgi targeting signal further, the transmembrane domain and the 24 adjacent amino acids in the cytosolic domain of NSvc2-N were swapped with the corresponding domain of NSvc2-C. As illustrated in Fig. 8S-U, this chimeric NSvc2-C(TMD_NCT_Ndel46)-YFP protein was also capable of localizing to the Golgi apparatus. Taken together, these data suggest that the ER-to-Golgi targeting signal resides in the C-terminal region (amino acids 269-315) of NSvc2-N, encompassing the 23-amino-acids transmembrane domain and 24 adjacent amino acids in the cytosolic tail.

DISCUSSION

In this study, using *N. benthamiana* as a model system we demonstrated here for the first time that the glycoproteins from an unenveloped *Tenuivirus* could target into Golgi bodies in plant cells. The RSV NSvc2-N glycoprotein alone targeted to the Golgi apparatus, while the NSvc2-C glycoprotein accumulated in the ER membrane in the absence of NSvc2-N. Upon co-expression, NSvc2-N was able to redirect NSvc2-C from the ER to the Golgi apparatus. Using the Sar1 or Arf1 dominant-negative mutants, we demonstrated that the targeting of NSvc2 glycoproteins to the Golgi apparatus was dependent on an active COP I or COP II

446 secretion pathway. We further revealed that the Golgi targeting signal mapped to a
447 region of the NSvc2-N protein (amino acids 269-315) encompassing the
448 23-amino-acids transmembrane domain (TMD) and the adjacent 24 amino acids of the
449 cytosolic tail.
450
451 The targeting of viral glycoproteins to the Golgi apparatus plays a pivotal role in the
452 formation of enveloped spherical particles for the viruses (animal- and plant-infecting)
453 in the *Bunyaviridae* family (15, 17, 35-41). Although RSV particle adopt long
454 filamentous morphology (20, 21), the subcellular targeting to the Golgi apparatus
455 seems to be a conserved mechanism between the unenveloped *Rice stripe tenuivirus*
456 and the enveloped viruses in *Bunyaviridae*. Why RSV glycoproteins do not facilitate
457 the formation of an enveloped spherical particle remains to be extensively
458 investigated in the future. It is interesting to note that despite the common
459 glycoprotein characteristics shared by RSV and viruses in the *Bunyaviridae*, all of the
460 viruses in the *Bunyaviridae* have larger size of glycoproteins than are found in RSV.
461
462 For TSWV, the type member of *Tospovirus* which is the only genus containing
463 plant-infecting viruses in the family *Bunyaviridae*, the glycoproteins forming the
464 surface spikes of the mature viral particle play an important role in insect transmission
465 (42). The key step where the virus enters the insect midgut cells is mediated by these
466 glycoproteins (42). RSV particles must also enter the midgut cells of *L. striatellus* to
467 complete their circulative-propagative transmission. The RSV-encoded glycoproteins

were predicted to have a similar role in vector transmission. Although the NSvc2 protein was not detected in the filamentous RSV particle, this protein may function as a bridge between the virus particle and recognition sites on the insect cell, as is seen, for example, with helper component-proteinase (Hc-Pro) of potyvirus (43). The targeting of RSV NSvc2 proteins to the Golgi apparatus could be an essential process for glycoprotein modification and maturation, allowing the attachment of the RSV RNP particle and subsequent vector transmission.

Zhao *et al.* (2012) reported that all of the RSV NSvc2 glycoproteins, including NSvc2-N, NSvc2-C and the full-length NSvc2 localized exclusively to the ER membrane in Sf9 insect cells (21). Our findings on the Golgi targeting of NSvc2 glycoproteins in *N. benthamiana* cells were different from those reported by Zhao et al. (2012) in Sf9 insect cells. The RSV NSvc2 glycoproteins may have different subcellular localization patterns in different systems. The NSvc2 glycoproteins target to the Golgi apparatus in plant cells, while they were arrested in the ER membrane in insect cells. These two different findings together lead to an interesting new concept that acquisition of RSV viral particle from plant host by *L. striatellus* insect vector may require glycoproteins which need to obtain glycosylation or similar modification in the Golgi apparatus whereas transmission of RSV viral particle from insect vector back into plant host may not require glycoproteins.

The leaf Golgi complex functions as a motile system that acquires products from a

relatively stationary ER system (28, 31). The glycoproteins of TSWV have shown to target into Golgi body using a tobacco protoplast system (44). However, the movement of the viral glycoproteins in the plant cell has not been shown previously. We demonstrated in this study that the targeted NSvc2 glycoproteins moved together with the Golgi stacks along the ER/actin network in *N. benthamiana* epidermal cells. The movement of the NSvc2-N glycoprotein together with the Golgi stacks in the *N. benthamiana* epidermal cells gives rise to an interesting hypothesis that the NSvc2-N could be acting as a mobile system for picking up NSvc2-C from the ER and transport it into the Golgi stacks. This hypothesis is consistent with the finding that the NSvc2-N protein accumulated in the Golgi stacks as early as 24 hpi, whereas the NSvc2-C protein alone remained consistently localized in the ER. NSvc2-C only began to accumulate in the Golgi apparatus at 48 hpi in the presence of NSvc2-N. The constant movement of NSvc2-N will continue to pick up NSvc2-C in the Golgi stacks over time.

RSV NSvc2-N was able to facilitate NSvc2-C transport from the ER to the Golgi apparatus. Export of proteins from the ER in plant cells has been suggested to occur through different routes (45-48). For ER-to-Golgi transport, a widely accepted pathway is based on the sequential action of COP II and COP I complexes (27). Our results showed that RSV NSvc2-N and the NSvc2-N::NSvc2-C complex migrate to the Golgi apparatus via the ERES and that Golgi targeting was strictly dependent on a functional anterograde traffic out of the ER to the Golgi or a retrograde transport route

512 from the Golgi apparatus, as over-expression of Sar1 (H74L) and Arf1 (T31N)
513 aborted NSvc2-N as well as NSvc2-N::NSvc2-C complex trafficking to the Golgi. In
514 the mammalian system, it has been demonstrated that the COPII coat recognizes and
515 selects export cargo into ERES vesicles (49). Our finding that the targeting of NSvc2
516 protein into Golgi via ERES suggests that COPII machineries, such as Sar1 or
517 Sec23-Sec24 complex, may be involved in selecting NSvc2 glycoproteins to target
518 into Golgi.

519

520 For viruses in the *Bunyaviridae* family, intracellular maturation and budding in the
521 Golgi complex is mediated by the targeting and accumulation of the viral
522 glycoproteins in this cellular compartment (17, 18, 35, 38-40). Previous work has
523 shown that the Golgi targeting signal of the TSWV and BUNV glycoproteins resides
524 in the transmembrane domain of the Gn protein, allowing for sufficient ER-exit and
525 transport to the Golgi (35, 36). However, the Golgi localization signal of RVFV was
526 mapped to a 48-amino-acid region of Gn containing the transmembrane domain and
527 the adjacent 28 amino acids of the cytosolic tail (17). Although UUKV is also a
528 phlebovirus, the Golgi localization signal for the UUKV glycoproteins resides in the
529 cytosolic tail of Gn (15, 50). In this study, we have mapped the Golgi targeting signal
530 of RSV to a region encompassing the transmembrane domain and the 24 adjacent
531 amino acids of the cytosolic tail of the N-terminus of NSvc2. Although the
532 tenuiviruses has very close relationship to the phleboviruses, our finding support that
533 the Golgi targeting motif of the RSV glycoprotein is more closely related to that of

RVFV, instead of UUKV glycoprotein.

In summary, our results presented here reveal that *Rice stripe tenuivirus* glycoproteins were able to target into Golgi apparatus in plant cells. Targeting of RSV glycoproteins into Golgi apparatus is mediated by the N-Terminal transmembrane domain and the adjacent cytosolic 24 amino-acids of NSvc2 in a COP I- and COP II-dependent manner. In light of the evidence from viruses in *Bunyavidae* that targeting into Golgi apparatus is important for the viral particle assembly and vector transmission, we propose that targeting of RSV glycoproteins into Golgi apparatus in plant cells represents a physiologically relevant mechanism in the maturation of RSV particle complex for insect vector transmission.

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682 **FIGURE LEGENDS:**

683 **FIG 1** Schematic diagrams of the viral constructs used for expression analysis (the
684 glycoprotein constructs are aligned below the precursor). Predicted cleavage sites
685 (scissor symbols) and amino acid positions are indicated. SS, TMD and CT refer to
686 the signal sequence, the transmembrane domain and the cytosolic tail, respectively.
687 SS_N and SS_C refer to the SS of NSvc2-N and NSvc2-C, respectively. TMD_N and
688 TMD_C refer to the TMD of NSvc2-N and NSvc2-C, respectively. CT_N and CT_C refer
689 to the CT of NSvc2-N and NSvc2-C, respectively. An intron of the potato ST-LS1
690 was inserted at the nucleotide position of 1182 on NSvc2. In all constructs, the YFP
691 fluorophore was fused in frame at the site of the stop codon.
692
693 **FIG 2** Subcellular localization of the NSvc2-N protein in *Nicotiana benthamiana* leaf
694 epidermal cells. (A) Immunoblot analysis of NSvc2-N-YFP fusion proteins expressed
695 by agroinfiltration in *N. benthamiana* leaves. The blots were probed using anti-YFP.
696 Empty vector (EV) was used as a negative control. Ponseau S was used as a loading
697 control. (B) Subcellular fractionation analysis of NSvc2-N-YFP fusion protein. The
698 soluble (S30) and microsomal (P30) fractions were isolated from agroinfiltrated
699 leaves of *N. benthamiana*. The membrane blots were probed using anti-YFP. (C-E)
700 The co-localization of the NSvc2-N-YFP (C) with the ER labeled by mCherry-HDEL
701 at 36 hpi (D). (E) Merged image of (C) and (D). (F-H) The co-localization of the
702 NSvc2-N-YFP (F) with the Golgi apparatus labeled by Man49-mCherry at 36 hpi (G).
703 The merged image illustrates the NSvc2-N protein targeted to the Golgi apparatus (H).

Scale bars, 20 µm.

FIG 3 Subcellular localization of the NSvc2-C protein in *Nicotiana benthamiana* leaf epidermal cells. (A) Western blot analysis of NSvc2-C-YFP fusion proteins expressed by agroinfiltration in *N. benthamiana* leaves. The blots were probed using anti-YFP. Ponseau S was used as a loading control. Empty vector (EV) was used as a negative control. (B) Subcellular distribution of NSvc2-C-YFP protein by fractionation analysis. The soluble (S30) and microsomal (P30) fractions were isolated from agroinfiltrated leaves of *N. benthamiana*. The membrane blots were probed using anti-YFP. (C-E) The co-localization of the NSvc2-C-YFP (C) with the ER labeled by mCherry-HDEL at 36 hpi (D). The merged image shows that NSvc2-C-YFP align well with the ER membrane (E). (F-H) The co-localization of the NSvc2-C-YFP (F) with the Golgi apparatus labeled by Man49-mCherry at 36 hpi (G). (H) Merged image of (F) and (G). Scale bars, 20 µm.

FIG 4 Subcellular localization of NSvc2-YFP in *Nicotiana benthamiana* leaf epidermal cells. (A) Immunoblot analysis of NSvc2-YFP fusion proteins (NSvc2-N and NSvc2-C-YFP glycoproteins were processed from its common glycoprotein precursor NSvc2-YFP) expressed by agroinfiltration in *N. benthamiana* leaves. The membrane blots were probed using anti-YFP. Ponseau S was used as a loading control. Empty vector (EV) was used as a negative control. (B) Subcellular distribution analysis of NSvc2-YFP protein by fractionation. The soluble (S30) and microsomal

(P30) fractions were isolated from agroinfiltrated leaves of *N. benthamiana*. The membrane blots were probed using anti-YFP. (C-E) Co-expression of the NSvc2-YFP (NSvc2-C-YFP was processed from this glycoprotein precursor) (C) with mCherry-HDEL (D) at 48 hpi. (E) Merged image of (C) and (D). (F-H) The co-localization of the NSvc2-YFP (NSvc2-C-YFP was processed from the glycoprotein precursor) (F) with the Golgi apparatus labeled by Man49-mCherry (G) at 48 hpi. The merged image shows the NSvc2-C protein targeted to the Golgi apparatus in the presence of NSvc2-N (H). Scale bars, 20 µm.

FIG 5 NSvc2 glycoproteins trafficking together with the Golgi stacks along the ER track in *Nicotiana benthamiana* leaf epidermal cells. (A-C) Time-lapse confocal images showing the movement of NSvc2-N-YFP (A) and the Golgi apparatus (B) labeled by Man49-mCherry at the times indicated. The position of the tracked signal is marked with an arrow. (C) Merged image of (A) and (B). (D-F) Time-lapse confocal images showing the movement of NSvc2-YFP (NSvc2-N and NSvc2-C-YFP were processed from the glycoprotein precursor NSvc2-YFP) (D) and the Golgi apparatus (E) at the times indicated. The position of the tracked signal is marked with an arrow. The merged images demonstrate that the NSvc2 proteins move together with the Golgi apparatus along the ER track (F). Scale bars, 20 µm.

FIG 6 ER-to-Golgi targeting of RSV NSvc2 glycoproteins depends on a functional COP II complex. (A-C) Confocal images of *Nicotiana benthamiana* epidermal cells

co-expressing NSvc2-N-YFP (A) and the COP II marker CFP-Sec24 at 36 hpi (B). (C) Merged image of (A) and (B). The arrows mark co-localization of NSvc2-N-YFP bodies with the ERES labeled with CFP-Sec24. (D-F) Co-expression of the dominant-negative mutant Sar1 (H74L) causes the redistribution of NSvc2-N-YFP (D) as well as the Golgi apparatus (E) back to the ER. (F) Merged image of (D) and (E). (G-I) Cells co-expressing NSvc2-N and NSvc2-C-YFP (from their common precursor NSvc2- YFP) (G) and the ERES labeled with CFP-Sec24 at 48 hpi (H). (I) Merged image of (G) and (I). (J-L) Co-expression of the dominant-negative mutant Sar1 (H74L) inhibits the transport of NSvc2-N and NSvc2-C-YFP (co-expressed from their common precursor NSvc2-YFP) to the Golgi complex. Scale bars, 20 µm.

FIG 7 ER-to-Golgi targeting of RSV NSvc2 glycoproteins depends on an active COP I complex. (A-C) Confocal images of *Nicotiana benthamiana* epidermal cells co-expressing NSvc2-N-YFP (A) and the COP I marker labeled with Arf1-CFP at 36 hpi (B). (C) Merged image of (A) and (B). (D-F) Co-expression of the dominant-negative mutant Arf1 (T31N) led to the retention of NSvc2-N-YFP (D) as well as the Golgi apparatus (E) in the ER at 48 hpi. (F) Merged image of (D) and (E). (G-I) Cells co-expressing NSvc2-YFP (G) and the COP I marker labeled with Arf1-CFP (H). (I) Merged image of (G) and (H). (J-L) Co-expression of the dominant-negative Arf1 (T31N) blocks transport of NSvc2-N and NSvc2-C-YFP (co-expressed from their common precursor NSvc2-YFP) to the Golgi complex. Scale bars, 20 µm.

770

771 **FIG 8** Golgi targeting signal analysis of truncated and chimeric NSvc2-N proteins.
772 **(**A-U) Confocal images of *Nicotiana benthamiana* epidermal cells co-expressing
773 Man49-mCherry with the truncated or chimeric proteins NSvc2-N del46-YFP (A-C),
774 NSvc2-N del63-YFP (D-F), $SS_NTMD_NCT_N$-YFP (G-I), $SSnTMD_N$-CT_Ndel46-YFP
775 (J-L), $SS_NTMD_NCT_N$del63-YFP (M-O), NSvc2-C(TMD_NCT_N)-YFP (P-R), and
776 NSvc2-C(TMD_NCT_Ndel46)-YFP (S-U), respectively, at 48 hpi. Scale bars, 20 µm.

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