



Japanese soil-borne wheat mosaic virus CP-RT protein associates with the endoplasmic reticulum network and mobile particles at the ER-Golgi interface during infection

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Abstract

Viruses transmitted by the obligate biotrophic parasite *Polymyxa graminis* cause substantial losses in agriculture worldwide. A better knowledge of the infection process may lead to the identification of novel strategies for virus control. The coat protein readthrough (CP-RT) protein or its homologs, present in all *P. graminis*-transmitted viruses, are transmission factors of the viruses. To better characterize CP-RT function, we modified an infectious Japanese soil-borne wheat mosaic virus (JSBWMV) cDNA clone to express fluorescent protein-tagged CP-RT and analyzed the protein during infection in the model host plant *Nicotiana benthamiana*. We found that JSBWMV CP-RT localized to and self-interacted at the endoplasmic reticulum (ER) network and in small cortical particles during infection. The particles moved along the ER network and associated with sub-ER sites, most likely ER import sites, as indicated by co-localization with Sar1-GTP:GFP and AtSYP72:GFP. Particles were still present after reabsorption of the Golgi into the ER network after Brefeldin A treatment, thus they were not an intrinsic part of the Golgi-ER compartment. In natural CP-RT deletion mutants, the localization to the ER network was lost, while localization to the particles was maintained. Consistent with earlier data our findings from transmission experiments in hydroponic barley culture indicate that an intact JSBWMV CP-RT may be important for virus transmission by the vector, as more wild type CP-RT forms compared to mutant CP-RT forms accumulated in newly infected plants. ER-association of CP-RT may thus be important for virus transmission and early infection processes.

Keywords Furovirus · Coat protein readthrough · Secretory pathway · Endoplasmic reticulum · Transmission · Virus movement

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Introduction

Japanese soil-borne wheat mosaic virus (JSBWMV) is a member of the species *Furovirus japonicum*, genus *Furovirus*, family *Virgaviridae* and infects barley and wheat. It is closely related to *Furovirus cerealis*, *Furovirus tritici* and *Furovirus chinense* (<https://ictv.global/report/chapter/virgaviridae/virgaviridae/furovirus>, last visited 25.02.2025). Furoviruses are transmitted by the obligate biotrophic spore forming root parasite *Polymyxa graminis* and cause extensive crop losses in agriculture (Kastirr et al. 2006; Rabenstein et al. 2011; Tamada and Kondo 2013; Kroese et al. 2020). The viral protein responsible for transmission is thought to be the coat protein readthrough (CP-RT) protein. However, the molecular mechanism how it acts in supporting virus transmission has not been resolved.

Polymyxa species belong to the order Plasmodiophorida, class Phytomyxea in the Rhizaria supergroup (Burki et al. 2010). Three species of Plasmodiophorids are recognized as important vectors of plant viruses, *P. graminis*, *Polymyxa betae* and *Spongospora subterranea* (Tamada and Kondo 2013). The viruses transmitted by these plasmodiophorids are rod shaped or filamentous particles belonging to the genera *Benyvirus* (Family *Benyviridae*), *Bymovirus* (Family *Potyviridae*), *Furovirus* (Family *Virgaviridae*), *Pecluvirus* (Family *Virgaviridae*) and *Pomovirus* (Family *Virgaviridae*). In addition to the rod-shaped furoviruses, *P. graminis* also transmits rod-shaped viruses from the genus *Benyvirus* and *Pecluvirus* and filamentous bymoviruses (Kühne 2009; Tamada and Kondo 2013). Interestingly, as bymo- and furoviruses co-occur in the same plants (Zhu et al. 2024; Ranabhat et al. 2025) it may not be unlikely that the same *P. graminis* spores can transmit both viruses. The sugar beet parasite *P. betae* transmits benyviruses and pomoviruses, while the potato pathogen *S. subterranea* transmits the pomovirus potato mop-top virus (PMTV) (Arif et al. 1995).

Plasmodiophorid zoospores infect host root epidermis or root hair cells by penetrating the cell wall and plasma membrane and injecting their cell content into the host cell cytoplasm (Kanyuka et al. 2003). Inside the host cell, plasmodiophorids then form a plasmodium, which further differentiates into resting spore clusters or into a zoosporangium, in which secondary zoospores form. In resting spores of the plasmodiophorid *S. subterranea*, PMTV-like particles have been detected (Merz 1995). Similarly, the benyvirus beet necrotic yellow vein virus (BNYVV) was detected in plasmodia, zoosporangia and mature zoospores of *P. betae* (Rysanek et al. 1992). Furthermore, RNA and movement protein as well as wheat spindle streak mosaic virus coat protein were detected in *P. graminis* resting spores, demonstrating internal association of the virus with the vector (Driskel et al. 2004). It is currently unknown how and at what stage of the infection

cycle virus transmission occurs; however, likely, transmission takes place at the zoosporangial stage, when secondary spores and viruses may be released into new cells, or at the plasmodium stage, when only a membrane barrier separates host and plasmodium cell.

Furoviruses are positive-sense single-stranded RNA viruses with a bipartite genome. The RNAs are capped and have a tRNA-like structure at their 3' end. RNA1 encodes the replication readthrough protein containing methyltransferase, helicase and RNA-dependent RNA-polymerase functions, a shorter protein lacking the RDRP domain and a movement protein (MP), presumably involved in the cell-to-cell movement of the virus. RNA2 encodes a 25 kDa protein of yet unknown function, the coat protein (CP), the CP-RT protein produced by translational readthrough of a leaky stop codon after the CP open reading frame (Shirako 1998; Yamamiya and Shirako 2000; Sun et al. 2013a) and a 19 K cysteine-rich silencing suppressor (Te et al. 2005; Sun et al. 2013b; Andika et al. 2016; Li et al. 2022).

The CP-RT protein represents a factor important for virus transmission by the plasmodiophorid vector. Several studies have reported the association of the CP-RT protein or homolog proteins in other plasmodiophorid-transmitted viruses with transmission and showed that mutations within the RT domain abolished vector transmission (Tamada et al. 1996; Reavy et al. 1998; Rochon et al. 2004; Tamada and Kondo 2013). Interestingly, the CP-RT domain is prone for deletion mutations as natural CP-RT deletion mutants are obtained from systemically infected field plants and upon experimental passaging. Tamada et al. (1996) conducted a study testing the transmissibility of different in-frame RT domain deletion mutants of BNYVV P75 and identified a motif in the C-terminal part of the RT domain that, if deleted, blocked virus transmission by its vector, *P. betae*. However, deletions within the C-terminal part of the protein did not impair virion assembly. Consistently, the C-terminal part of the JSBWMV CP-RT protein was not essential for virion formation and stable JSBWMV CP-RT mutants lacking a part of the RT region were still infectious and able to move systemically upon mechanical inoculation in wheat (Yamamiya and Shirako 2000; Yamamiya et al. 2005). Within the CP-RT proteins or homolog proteins from different plasmodiophorid-transmitted virus genera conserved, predicted transmembrane domains (TMs) which appear to be important for vector-mediated transmission of the virus exist (Adams et al. 2001). The TMs have been proposed to play a role in transporting the virus across the plasmodium-membrane; however, direct evidence for this hypothesis is still missing. Reassortment experiments of JSBWMV and SBWMV from the USA have been conducted (Miyaniishi et al. 2002) and demonstrated the close relatedness of the two virus species *Furovirus japonicum* and *Furovirus tritici*. Apart from their role in virus transmission, CP-RT

proteins have been shown to constitute structural components of viruses (Haeberlé et al. 1994; Cowan et al. 1997; Xu et al. 2002). However, if the function of CP-RT as a structural virus component is linked to its role in virus transmission and if the CP-RT protein has additional roles in virus movement has not been addressed.

Here, we set out to further illuminate CP-RT function during virus transmission and movement by investigating the subcellular localization of the JSBWMV CP-RT protein during infection with an infectious cDNA clone, which expressed GFP or RFP fused to the CP-RT protein. CP-RT localized to the endoplasmic reticulum (ER) network and to small, cortical particles, which were mobile along the ER network. CP-RT self-interacted at the ER network and in the small particles. The particles labeled sub-sites of the ER, which coincided with ER import sites (ERIS), but were not part of the ER-Golgi interface as they were resistant to Brefeldin A treatment. The localization at the ER network, but not at particles, was lost in naturally occurring CP-RT deletion mutants that spanned the predicted TMs. Transmission experiments showed that wild type (WT) CP-RT forms occurred to higher extent compared to mutant forms in newly infected plants, suggesting that the WT CP-RT forms and ER-network association may be important for transmission and/or early events of virus movement.

Materials and methods

Plant material and cultivation

Chenopodium quinoa, wild type *N. benthamiana* and the transgenic line *N. benthamiana* 16c (Ruiz et al. 1998) were grown with 16 h day length and 20–22 °C in the greenhouse. After mechanical infection with in-vitro transcribed virus RNA, plants were cultivated at 17 °C, as efficient virus replication requires temperatures below 20 °C (Ohsato et al. 2003) and JSBWMV infection develops well at 17 °C in mechanically inoculated *C. quinoa* leaves (Yamamiya and Shirako 2000; Strauch et al. 2025). *Agrobacterium tumefaciens*-infiltrated plants were kept at 24 °C. Barley (*Hordeum vulgare*) in hydroponic culture were grown with 16 h day length and 17 °C in the greenhouse. Barley plants, which were directly grown in infectious field soil, were grown at 12 °C with 12 h day length. All experiments were conducted with 4–6 week old plants, except otherwise stated.

Viruses

JSBWMV strain JT was obtained in form of infectious clones, plasmids pJS1 and pJS2, containing JSBWMV cDNA for RNA1 and RNA2 (Yamamiya and Shirako 2000), were kindly

provided by Hideki Kondo (Okayama University, Japan). JSBWMV-Bornum (Rabenstein et al. 2011; Golyaev et al. 2019) was obtained from field plants in Bornum am Elm, Lower Saxony, Germany.

Plasmid construction

PCRs were performed with Phusion polymerase (NEB), according to the manufacturer's instructions. Plasmids pJS1 and pJS2, containing JSBWMV cDNA for RNA1 and RNA2 (Yamamiya and Shirako 2000), were kindly provided by Hideki Kondo (Okayama University). The Gateway system (Invitrogen) was used according to the manufacturer's instructions for cloning JSBWMV CP-RT (NCIB: NC_038851.1), JSBWMV-Bornum CP-RT (NCIB: KU236377.1, Rabenstein et al. 2011) and *A. thaliana* *AtSYP72* (TAIR: AT3G45280) into expression vectors suitable for agrobacterium-mediated leaf infiltration. The JSBWMV CP-RT gene was amplified by using pJS2 as template and primers with flanking *attB*-sites. Forward Primer 5'-G GGGACAAGTTTGTACAAAAAAGCAGGCT-TAAAC ATGGTGTGAAAAATGGTTACAC and reverse primer 5'-GGGGACCACTTTGTACAAGAAAGCTGGGT-CG GACGCCATAGGCCCACTA were used. The JSBWMV-Bornum CP-RT gene and its mutants were amplified using cDNA from virus infected barley plants as template. Forward Primer 5'-GGGGACAAGTTTGTACAAAAAAGCA GGCTTAAACATGGCTGTAAAGAATGGTTACAC and reverse primer 5'-GGGGACCACTTTGTACAAGAAAGC TGGGTTCGACGCCATAGGCCTACTAG were used. The *AtSYP72* coding sequence was amplified using *A. thaliana* cDNA from young seedlings as template. Forward primer 5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTTAA ACATGCCGGTCATTGATATC and reverse primer 5'-G GGGACCACTTTGTACAAGAAAGCTGGGTTCGTTA GAGCATTGTAAATGTAAG were used. The PCR products with flanking *att*-sites were recombined into the Gateway donor vector pDONR/Zeo (Invitrogen) to create entry vectors for JSBWMV CP-RT, JSBWMV-Bornum CP-RT and the CP-RT mutants (CP-RT^{mutA} and CP-RT^{mutB}) and *AtSYP72*. Following sequence confirmation, the entry vectors were used for recombination with pB7FWG2 to create the respective GFP expression vectors; while pH7RWG2 was used to create the respective RFP expression vectors (Karimi et al. 2002). Entry and expression vectors for JSBWMV CP-RT were transformed into *E. coli* TOP10, while those for *AtSYP72*, JSBWMV-Bornum CP-RT and the respective mutants were transformed into *E. coli* DH5α. Plasmid propagation was done in liquid LB medium at 37 °C, supplemented with antibiotics for plasmid selection. Plasmid DNA was extracted from overnight cultures using the NucleoSpin Plasmid kit (Macherey-Nagel). PCR

products were extracted from agarose gels by cutting out and subsequent DNA purification using the PCR Clean-up Mini kit (Macherey-Nagel).

For modification of the JSBWMV cDNA clone, the CP-RT:FP sequences were amplified by PCR from the expression vectors pB7-CP-RT:GFP and pH7-CP-RT:RFP to add *Bgl*III restriction sites (bold) to the 3'-end of GFP and RFP coding sequences. Forward primer 5'-GAGTTAGTGGA GAAGTGAATTG and reverse primer 5'-GAAGATCTT TACTTGTACAGCTCGTCC were used to create pJS2-CP-RT:GFP, while forward primer 5'-TGGCAGCTGAGAACC TAGAG and reverse primer 5'-GAAGATCTTTAGGCGCC GGTGG were used to create pJS2-CP-RT:RFP. The resulting PCR products were fully digested with *Bgl*III (NEB) and purified with the PCR Clean-up Mini kit (Macherey-Nagel). The digested and purified DNA fragments were ligated with T4 ligase (NEB) into pJS2, which was partially digested in advance with *Bgl*III (NEB) for approximately 30 min. After successful ligation, resulting plasmids pJS2-CP-RT:GFP and pJ2-CP-RT:RFP contained JSBWMV RNA2 cDNA with CP-RT fused to GFP or RFP and a duplicated sequence of 279 nt at the 3' end of the RT, as it has been shown that the 3'-terminal region of the JSBWMV RT gene has a major function in cis for expression of the 19 K cysteine-rich silencing suppressor, which is essential for infection (Yamamiya et al. 2005). Plasmids were transformed into *E. coli* TOP10 for plasmid propagation and storage. All inserts and plasmid modifications were verified by nucleotide sequencing (Eurofins Genomics). Primers used for sequencing pJS2-CP-RT:GFP and pJS2-CP-RT:RFP are listed in Table S1.

In-vitro transcription and virus inoculation

DNA from pJS1, pJS2 and the modified plasmids pJS2-CP-RT:GFP, pJS2-CP-RT:RFP was linearized with *Spe*I (NEB), directly downstream of the 3' terminus of JSBWMV RNA1 and RNA2 and purified with the PCR Clean-up Mini kit (Macherey-Nagel). The linearized plasmids were used as template for in-vitro transcription of JSBWMV RNA1, RNA2 and the modified JSBWMV-CP-RT:GFP and JSBWMV-CP-RT:RFP RNA2. The SP6-driven transcription reactions were performed for 3 h at 37 °C with the SP6 RiboMAX™ Large Scale RNA Production System (Promega), using approximately 1 µg template DNA, in the presence of 5 mM Adenosine, Cytosine, Uracil, 0.6 mM Guanosine and 2.67 mM m7G-cap analog (Promega) per reaction. The quantity and integrity of in-vitro produced RNA transcripts was ascertained in 1% (w/v) agarose gels by electrophoresis and staining with Midori-Green (Biozym). Transcripts of RNA1 and either RNA2 or the modified RNA2 were mixed in equal volumes and dissolved 1:4 (v/v) in 50 mM glycine and 50 mM K₂HPO₄, pH 9.2 (Yamamiya and Shirako 2000). Drops of the viral RNA inoculum were

pipetted onto the leaf surface of *C. quinoa* or *N. benthamiana* and rub-inoculated in the presence of Celite®. After 30 min, rub-inoculated leaves were rinsed with tap water to remove the Celite®. Inoculated plants were kept at 17 °C.

Transient protein expression via agroinfiltration

Agrobacterium tumefaciens-containing expression vectors for the expression of fluorescent proteins and the tomato bushy stunt virus (TBSV) 19 K silencing suppressor (Hamilton et al. 2002) in plants were grown at 28 °C in liquid 2xYT (2x yeast extract tryptone medium) medium supplemented with antibiotics, harvested by centrifugation, resuspended in 10 mM MES with 10 mM MgCl₂ and set to a certain optical density at 600 nm (OD₆₀₀) for leaf infiltration. A final OD₆₀₀ of 0.16 for TBSV 19 K and 0.33 for all other bacteria suspensions was used. For agroinfiltration into virus infection sites, the final OD₆₀₀ for TBSV 19 K was 0.008 and 0.016 for fluorescent markers. The bacteria suspensions were infiltrated into the abaxial side of *N. benthamiana* leaves using a syringe without needle. Infiltrated plants were cultivated at 24 °C for >48 h, except otherwise stated. To identify subcellular organelles in virus infection sites, plants transiently expressing AtSYP72:RFP or Man1:RFP were transferred from 24 °C to 17 °C and the agro-infiltrated leaves were rub-inoculated with in-vitro transcribed JSBWMV RNA. JSBWMV infected plants were cultivated at 17 °C, as the virus replicates efficiently below 20 °C (Ohsato et al. 2003). Local infection sites were observed after at least three days by confocal laser scanning microscopy.

Brefeldin A (BFA) treatment

Stock solutions containing 1 mg/mL BFA in DMSO were diluted in 10 mM MgCl₂ to a concentration of 20 µg mL⁻¹. 20 µg mL⁻¹ DMSO served as negative control. *N. benthamiana* leaves expressing CP-RT:FP together with fluorescent marker proteins were infiltrated into the abaxial side of the leaf with the prepared inhibitor solutions, using a syringe without needle.

Infection of barley plants with wild type JSBWMV

To establish infections originating from *P. graminis* spores containing JSBWMV, seeds of barley cv. Effi or cv. Kathleen were directly sown on field soil collected from Bornum am Elm (Lower Saxony, Germany), which contains infectious *P. graminis* spores carrying JSBWMV. Three months after sowing, leaf material was tested for JSBWMV infection by enzyme linked immunosorbent assay (ELISA). To infect barley with JSBWMV-Bornum via rub-inoculation, infectious sap was prepared from ELISA-positive plants. The sap was prepared from leaf material in a pre-chilled mortar with phosphate buffer (10 mM K₂HPO₄/KH₂PO₄) and Celite®, using a pestle.

Ten-day old healthy barley cv. Kathleen or cv. Jakubus, grown in potting soil, were inoculated by rubbing the leaf surface with freshly prepared sap. To investigate virus transmission, healthy barley cv. Effi and cv. Kathleen were cultivated in the presence of *P. graminis* and JSBWMV-Bornum infected barley plants in a hydroponic culture system. Hydroponic culture boxes contained Hoagland medium. For each condition cv. Effi and cv. Kathleen, respectively, cultured for two or eight months in infectious field soil, separate culture containers were used. Seven source plants were inserted into each container after extensively washing the roots of the plants with tap water. Bait plants were germinated on tissue paper for three days before insertion into the hydroponic culture system. After five weeks of hydroponic culture, the bait plants were sampled. Roots were washed with tap water and RNA was extracted from roots and leaves, respectively. RT-PCR was performed to analyze CP-RT forms accumulating in the bait plants.

RNA extraction, synthesis of cDNA and PCR

Total RNA was extracted from barley leaves infected with JSBWMV-Bornum. 100 mg leaf material ground in liquid nitrogen using a TissueLyser (Qiagen) was processed with the RNeasy Plant Mini kit (Qiagen). Alternatively, RNA was extracted from roots and leaves of infected barley plants using Nucleo-Zol (Macherey-Nagel). cDNA was synthesized using random primers and reverse transcriptase (ProtoScript First Strand cDNA Synthesis Kit, NEB). All kits were used according to the manufacturer's instructions. PCR was conducted using primers SBBMV_GW_CPRT_For1 5'-GGGGACAAGTTTGTACAA AAAAGCAGGCTTAAACATGGCTGTAAAGAATGGTTA CAC and SBBMV_GW_CPRT_Rev1 5'-GGGGACCACTT TGTACAAGAAAGCTGGGTCCGACGCCATAGGCCTAC TAG in a PCR mix containing 1x Taq buffer (NEB), 200 µM dNTPs, 0.2 µM SBBMV_FW and SBBMV_RV primer, and 1.25 U Taq polymerase (NEB). PCR conditions were 5 min at 95 °C, 35 cycles with 30 s at 95 °C, 30 s at 50 °C, 2.5 min at 72 °C, followed by a final elongation for 10 min at 72 °C. To control the quality of the cDNA, each cDNA was submitted to a control PCR using primers against elongation factor 1 alpha (Jarošová and Kundu 2010) and the following PCR conditions: 2 min at 95 °C, 35 cycles of 15 s at 95 °C, 30 s at 55 °C and 15 s at 72 °C, followed by a final elongation of 10 min at 72 °C.

Detection of JSBWMV infection in barley and *N. benthamiana*

JSBWMV was detected in leaf extract prepared from infected barley and *N. benthamiana* plants by double-antibody sandwich ELISA (DAS-ELISA). Microtiter plates (Nunc Maxi-Sorp™, Invitrogen) were coated with IgG against JSBWMV (antibody: JKI SBBMV 92/2), which was diluted 1:100 in

coating buffer (15 mM Na₂CO₃, 35 mM NaHCO₃, 31 mM NaN₃, pH=9.6). 100 µL of the diluted antibody solution was placed into each well and incubated for 3–4 h at 37 °C. After coating, the plate was washed three times with wash buffer (phosphate-buffered saline with 0.2% Tween® 20 (PBST)). Leaf material was homogenized in sample buffer (PBST, 2% (w/v) polyvinylpyrrolidone, supplemented with 0.2% (w/v) dried egg albumin) using mortar and pestle. Subsequently, the leaf extract was diluted 1:15 in sample buffer and 100 µL of sample loaded in duplicate into the wells of microtiter plates, followed by overnight incubation in the refrigerator. The next day, the plates were washed three times with wash buffer. Alkaline-phosphatase-coated secondary antibody (antibody: JKI SBBMV 92) was diluted 1:1000 in sample buffer supplemented with 0.2% (w/v) dried egg albumin and added to the wells of the microtiter plates. After incubation for 3 h at 37 °C and three rounds of washing with wash buffer (PBST), 100 µL of substrate (1 mg/mL 4-nitrophenylphosphate in Diethanolamine-buffer (9.7% (w/v) diethanolamine, pH=9.8)) was added to each well of the microtiter plate and incubated for 1 h at room temperature. Absorbance was recorded at 405 nm after 1 h at room temperature (20–25 °C) with a Tecan Sunrise plate reader (Tecan Group AG). Buffer control samples served as blank control. Healthy and infected negative and positive control samples were included.

Transmission electron microscopy (TEM)

JSBWMV and JSBWMV-CP-RT:GFP infection sites were excised from inoculated *C. quinoa* leaves 14 days post inoculation (dpi) and homogenized in phosphate buffer pH=7 containing 2% polyvinylpyrrolidone, 0.2% sodium sulfite and 0.05% sodium azide. For immune electron microscopy, pioloform coated nickel grids with a carbon layer were incubated with 1:1000 diluted antiserum (AS) against SBWMV-CP (JKI-EP-EM AS1618) for 5 min. After washing with phosphate buffer, grids were incubated with plant extract overnight. The grids were washed and incubated with the antiserum diluted 1:50. A final washing step used ultraclean water before negative staining with 1% Uranyl acetate and drying of the grids. Dried grids were analyzed at 80 kV in a Tecnai Spirit G2 Twin electron microscope (FEI at Thermo Fisher Scientific) equipped with a tungsten cathode. Images were taken at 80 kV using a 2 K Veleta digital camera.

Confocal laser scanning microscopy and FRET-FLIM analysis

Fluorescent infection sites and transiently expressed fluorescent proteins were visualized with a Leica DM6 microscope (Leica microsystems) equipped with the TCS SP8 multiphoton system for confocal image acquisition. Cells were observed

under the HC PL FLUOTAR 10x/0.32 dry or HC PL APO 63x/1.20 CORR CS2 water immersion objective. Excitation of GFP derivatives was achieved with a 488 nm argon laser, while RFP derivatives were excited using a 561 nm diode pumped solid state laser. Emitted fluorescence signals were captured with an acousto-optical beam splitter (AOBS), allowing a detection of GFP fluorescence between 500 and 550 nm and red fluorescent signals from 590 to 640 nm. The pinhole was set to AU=1.0. The images were taken and processed with the Leica Application Suite X (Leica microsystems).

FRET-FLIM was performed as described in (Brandner et al. 2008) and (Boutant et al. 2010) with a home-built two-photon system. To gain an appropriate photon statistics for the fluorescence decays, the samples were scanned continuously for 45 s. Data analysis was performed with the software package (SPCIMAGE V2.8; Becker & Hickl), which uses an iterative reconvolution method to recover the lifetimes from the fluorescence decays.

The following formula was used to calculate the FRET-efficiency E .

$$E = \left(\frac{R_0^6}{R_0^6 + R} \right) = 1 - \frac{\tau_{fret}}{\tau_{free}}$$

In this formula, R_0 refers to the Förster radius, R is the distance between the donor and the acceptor, τ_{fret} represents the lifetime of the donor in presence of the acceptor, τ_{free} reflects the lifetime of the donor in absence of the acceptor. The FRET efficiency reflects the distance between the donor and the acceptor proteins. The 5% threshold applied in our FRET-FLIM experiments corresponds to an empirical confidence threshold, established beyond instrumental noise. It ensures that the observed variation in fluorescence lifetime reflects a real molecular interaction, rather than a simple experimental fluctuation. Thus, this 5% threshold constitutes a criterion of reliability and specificity that guarantees the validity of the measured interaction signal.

Results

The JSBWMV CP-RT protein expressed from an infectious virus clone localizes to the ER network and to ER-associated particles

To investigate the subcellular localization of JSBWMV CP-RT during infection, we modified the RNA2 of an infectious clone of JSBWMV to express the CP-RT protein fused to GFP or RFP. The clone was infectious on *N. benthamiana*

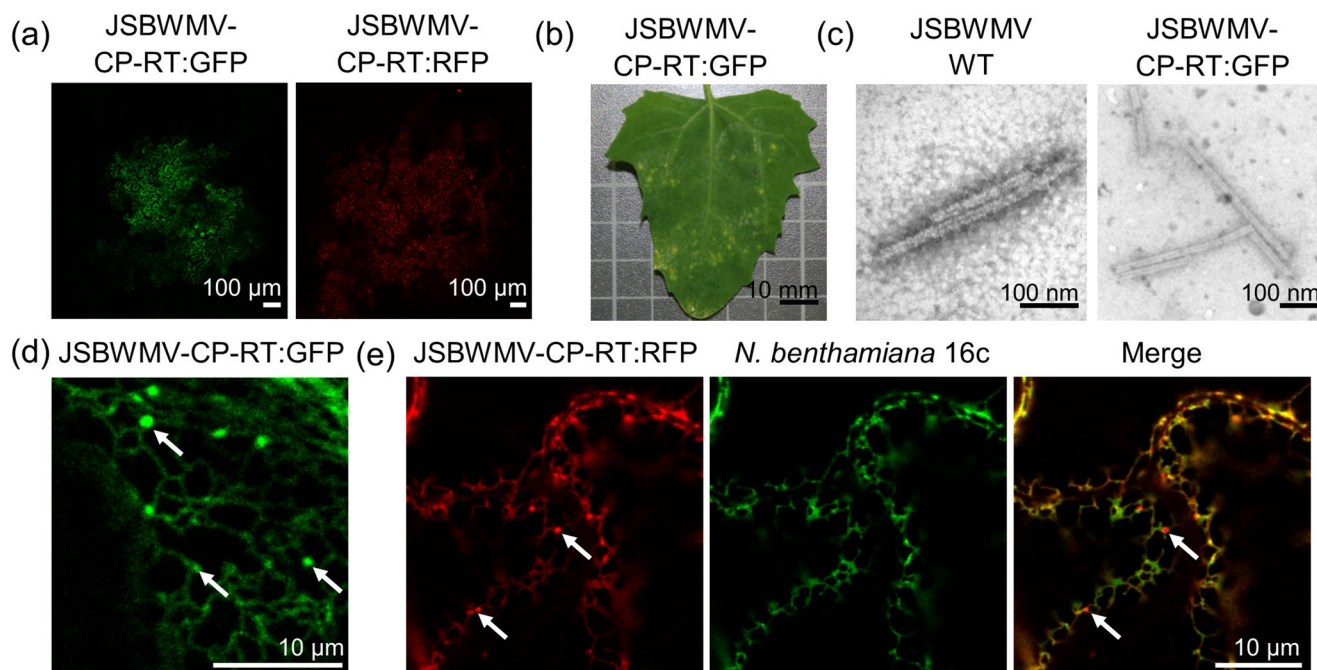


Fig. 1 Infection of *N. benthamiana* and *C. quinoa* with fluorescent protein-tagged JSBWMV. **(a)** Fluorescent infection site developed on a *N. benthamiana* leaf four and six days after JSBWMV-CP-RT:GFP (left panel) or JSBWMV-CP-RT:RFP (right panel) infection, respectively. **(b)** *C. quinoa* leaf rub-inoculated with JSBWMV RNA1 and JSBWMV-CP-RT:GFP RNA2 14 days post inoculation (dpi). Chlorotic spots indicate infection sites. **(c)** Immunoblot images of JSBWMV wild type (WT) and JSBWMV-CP-RT:GFP particles in crude extract from infected *C. quinoa* leaves.

(d and e) Fluorescent protein-tagged CP-RT labels a network-like structure and dot-like particles during infection. **(d)** CP-RT:GFP fluorescence in JSBWMV-CP-RT:GFP infected *N. benthamiana* leaf epidermal cells 4 dpi. **(e)** CP-RT:RFP fluorescence in JSBWMV-CP-RT:RFP infected *N. benthamiana* 16c leaf epidermal cells 6 dpi. Left panel, RFP channel; middle panel, GFP channel; right panel, overlay image of the RFP and GFP channels. Arrows point to dot like particles labelled by CP-RT:GFP or CP-RT:RFP, respectively

after rub-inoculation of the in-vitro transcribed RNA as multicellular fluorescent infection sites were observed from three days after infection (Fig. 1a). In *C. quinoa* leaves, rub-inoculation with RNA in-vitro transcribed from pJS1 and pJS2-CP-RT:GFP revealed symptom development in the form of numerous chlorotic lesions on the inoculated leaves 14 days post infection (Fig. 1b). Similar symptoms were described for mixtures of pJS1 and pJS2-derived RNAs inoculated onto *C. quinoa* (Yamamiya and Shirako 2000). Formation of virions similar to wild type virions was confirmed by electron microscopy of *C. quinoa* leaves infected with JSBWMV-CP-RT:GFP (Fig. 1c), suggesting that introduction of the fluorescent protein tag at the C-terminus of CP-RT did not interfere with virion formation.

To begin uncovering the role of the CP-RT protein *in planta*, we examined the subcellular localization of CP-RT:GFP and CP-RT:RFP expressed from the virus genome in infected *N. benthamiana* leaf cells. Within fluorescent infection sites, the CP-RT protein localized to a net-like

structure and small dots in the cell cortex (Fig. 1d and e). No difference was seen within and between fluorescent infection sites and between JSBWMV-CP-RT:GFP and JSBWMV-CP-RT:RFP. Using HDEL:GFP expressing *N. benthamiana* 16c (Ruiz et al. 1998), we identified the net-like structure as ER (Fig. 1e). The dot-like particles formed by CP-RT:GFP and CP-RT:RFP associated with the ER, mainly at the ER vertices, but did not co-localize with the ER network. Similar to its localization in virus infection sites, CP-RT:GFP and CP-RT:RFP ectopically expressed from a 35S promoter after agroinfiltration localized to the ER and ER-associated dot-like particles, which moved together with the ER (Fig. S1).

To investigate the nature of the dot-like particles, we expressed the ERIS marker AtSYP72:RFP (Lerich et al. 2012) (Fig. S2) in JSBWMV-CP-RT:GFP infection sites. AtSYP72:RFP exhibited the described dot-like distribution for AtSYP72 at the ER and a subset of AtSYP72:RFP particles colocalized with JSBWMV-CP-RT:GFP particles during infection (Fig. 2a). To further explore a localization

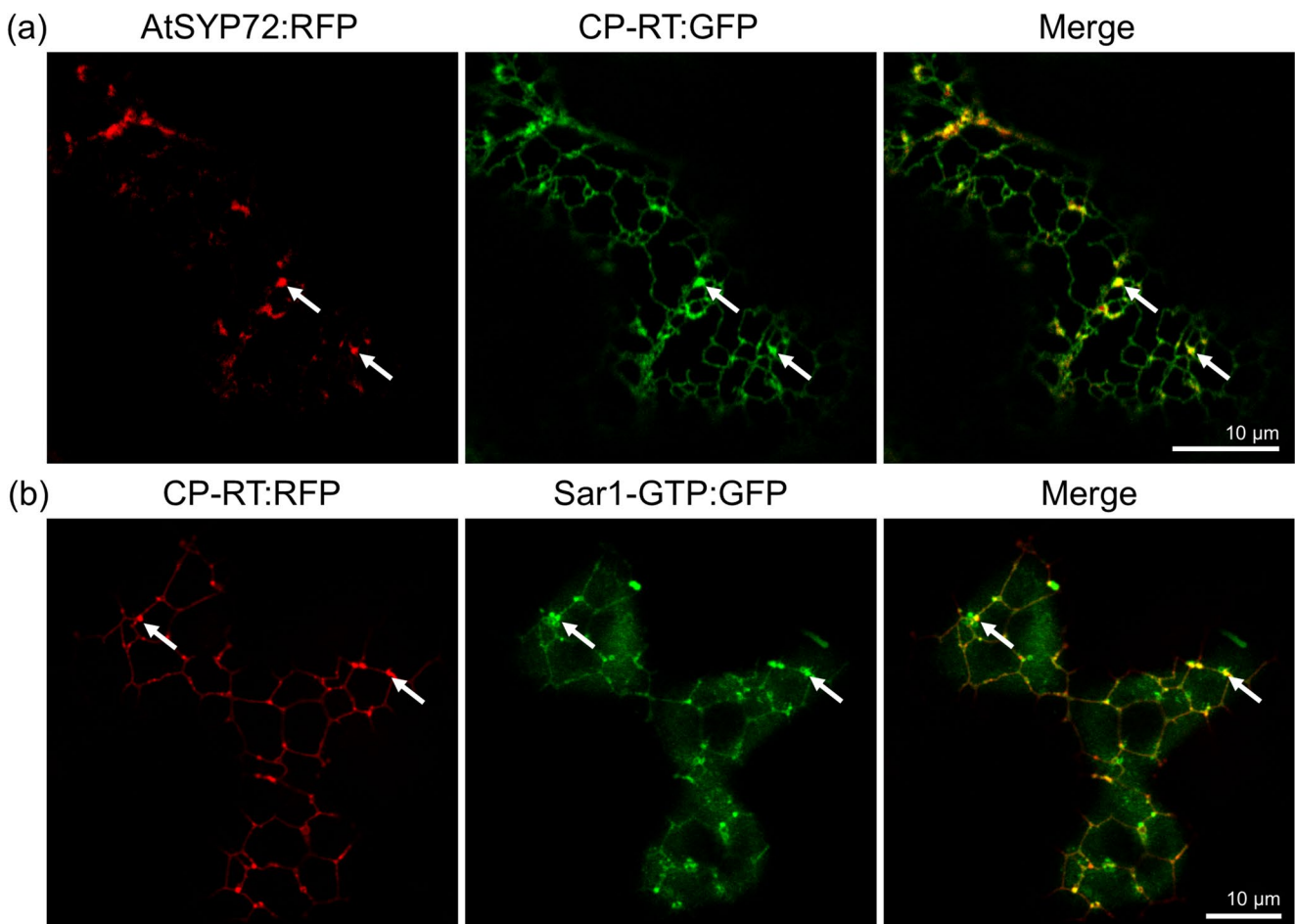


Fig. 2 Co-localization of JSBWMV-CP-RT:GFP and CP-RT:RFP with sub-ER sites. **(a)** Co-localization of the ERIS marker AtSYP72:RFP with CP-RT:GFP in JSBWMV-CP-RT:GFP infection sites on *N. benthamiana* leaves 6 dpi. **(b)** Co-localization of CP-RT:GFP and GTP-

locked Sar1:GFP (Sar1-GTP:GFP) in *N. benthamiana* leaf cells three days after agroinfiltration. Arrows mark co-localization in dot-like particles. Left panels, RFP channel; middle panels, GFP channel; right panels, overlay image of the RFP and GFP channels

of CP-RT at the ER-Golgi interface, we co-expressed CP-RT:RFP together with the GTP-locked Sar1 fused to GFP (Sar1-GTP:GFP), which impairs COPII vesicle formation for ER-to-Golgi transport and localizes to distinct dot-like ER sites and the ER network (Fig. S2) (daSilva et al. 2004; Osterrieder et al. 2010). CP-RT:RFP-labeled particles colocalized with Sar1-GTP:GFP dots (Fig. 2b). Thus, we concluded that the CP-RT particles represent sites at the ER involved in sorting and traffic through the ER-Golgi pathway.

Moreover, high expression levels of Sar1-GTP:GFP led to the appearance of membranous ring structures at the ER, which were often located at the vicinity of the CP-RT:RFP labeled particles (Fig. 2b). These rings might be a consequence of inhibited COPII vesicle formation; ring-like structures have also been observed upon expression of YFP-tagged wild type Sar1 in tobacco epidermal cells and were assumed to represent sites where new Golgi bodies form (daSilva et al. 2004). However, the appearance of ring-like structures may

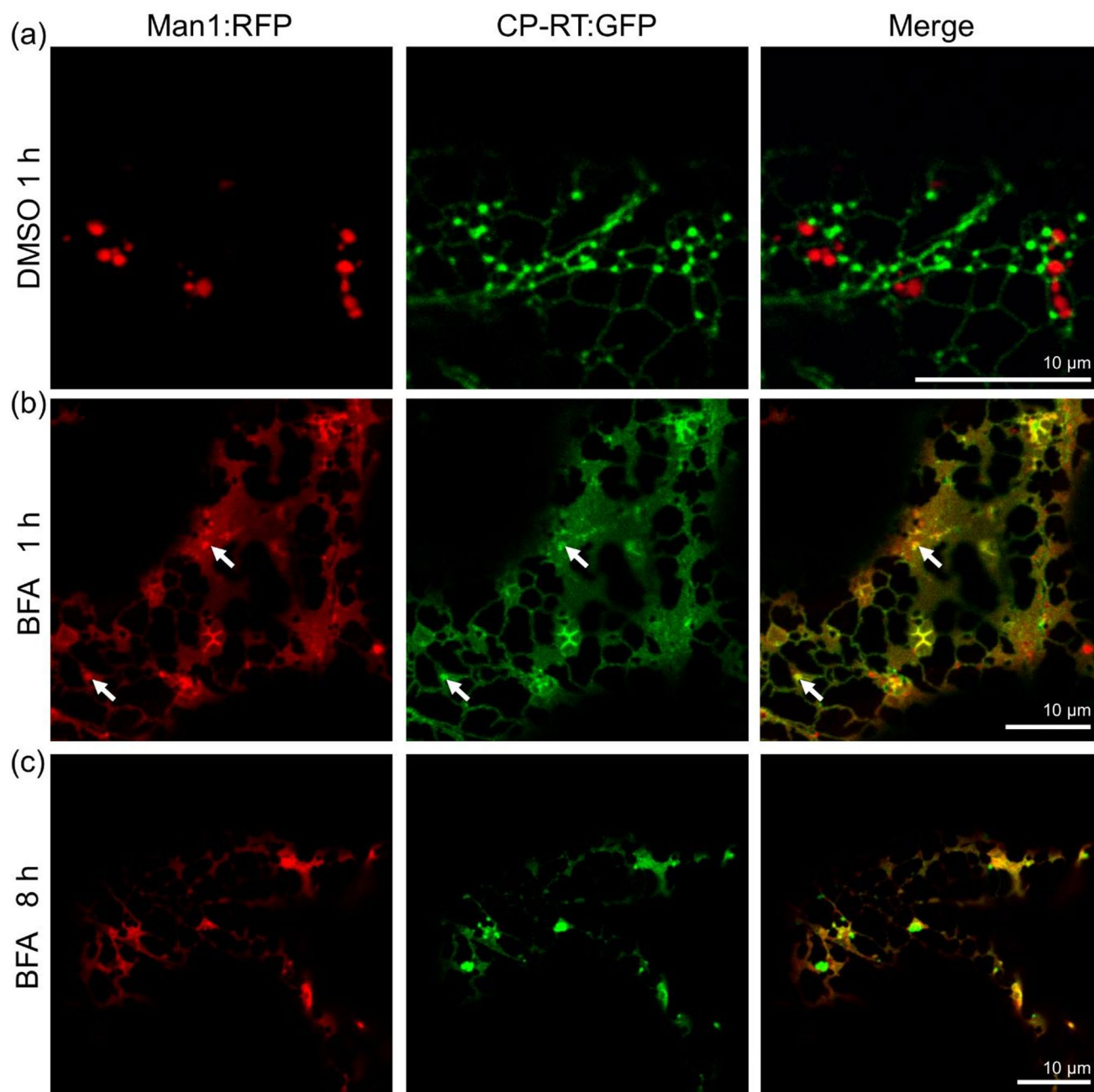


Fig. 3 Effect of BFA treatment on CP-RT:GFP and CP-RT:RFP localization. **(a, b)** CP-RT:GFP was ectopically co-expressed with Man1:RFP after infiltration of DMSO **(a)** or $20 \mu\text{g mL}^{-1}$ BFA 1 h prior to imaging. Arrows in **(b)** mark residual Golgi stacks colocalizing

with CP-RT:GFP after BFA treatment. **(c)** 8 h after BFA treatment, CP-RT:RFP still localized to dot-like particles at the surface of the hybrid compartment

also indicate the induction of alternative transport routes by inhibition of the secretory pathway.

To further investigate the involvement of the secretory pathway, we treated cells ectopically expressing CP-RT:GFP and the Golgi marker Man1:RFP with BFA, an inhibitor of ER-derived vesicle formation (Nebenführ et al. 2002). Under non-disturbed conditions or in DMSO-infiltrated control cells, no co-localization of CP-RT:GFP particles with Man1:RFP labelled Golgi was observed and Golgi exhibited its normal, dotted pattern (Fig. 3a and Fig. S3). By contrast, BFA treatment led to the reabsorption of the Golgi marker Man1:RFP into the ER approximately 1 h after BFA treatment (Fig. 3b). Interestingly, 1 h after BFA treatment, remaining Golgi colocalized with the dot-like CP-RT particles at the ER, confirming our suggestion, that the particles might mark ERIS (Fig. 3b). At later time points after BFA treatment, CP-RT dot-like particles were still present and localized in proximity to the ER-Golgi hybrid compartment (Fig. 3c).

JSBWMV CP-RT forms oligomers

The formation of the ER-associated dot-like particles during virus infection and ectopic expression, respectively, suggested that CP-RT might oligomerize. Thus, we conducted

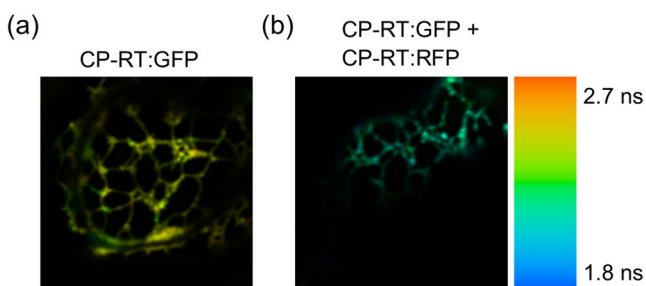


Fig. 4 FRET-FLIM of CP-RT:GFP in *N. benthamiana* epidermal cells. Fluorescence lifetime images of CP-RT:GFP are shown in a color code ranging from 1.8 ns (blue) to 2.7 ns (red). **(a)** Lifetime image for CP-RT:GFP expressed alone. **(b)** Lifetime image for CP-RT:GFP in the presence of CP-RT:RFP. Co-expression of CP-RT:GFP with CP-RT:RFP reduces the fluorescence lifetime of CP-RT:GFP. The lifetime values were measured separately in the ER network and dot-like particles. Pictures were taken three to four days after agroinfiltration

fluorescence resonance energy transfer (FRET)-fluorescence lifetime imaging microscopy (FLIM) experiments with ectopically expressed CP-RT:GFP and CP-RT:RFP to analyze the oligomerization and to spatially resolve self-interaction of the protein. FRET-FLIM revealed a fluorescence lifetime of CP-RT:GFP of 2.43 ± 0.05 ns when expressed alone and a drop in fluorescence lifetime to 2.05 ± 0.08 ns in the presence of CP-RT:RFP as acceptor, when the protein was localized at the ER network (Fig. 4; Table 1). The fluorescence lifetime of CP-RT:GFP in the dot-like structures was 2.42 ± 0.04 ns. A reduction in lifetime to 2.1 ± 0.11 ns was observed in presence of CP-RT:RFP. These data indicate that CP-RT oligomerizes at the ER and in the dot-like structures.

Mutations naturally occurring in the CP-RT protein affect ER localization, but not localization to dot-like structures

The CP-RT protein and its homologues from other plasmodiophorid-transmitted viruses are known to accumulate deletion mutations upon prolonged cultivation and upon mechanical transmission of the virus (Tamada and Kusume 1991; Reavy et al. 1998; Yamamiya and Shirako 2000; Yamamiya et al. 2005). Remarkably, independently occurring mutations in the CP-RT sequence share common features, as usually the predicted TMs within the CP-RT are deleted while the CP-RT C-terminus is maintained. However, while wild type CP-RT appears to be important for transmission by *P. graminis*, it is not clear whether the CP-RT mutants serve a function during infection. We thus generated such mutants for ectopic expression in *N. benthamiana* as fluorescent protein fusion and analyzed their subcellular distribution. To obtain the deletion mutants, we cultivated barley plants in JSBWMV-containing soil from Bornum (Germany) (Rabenstein et al. 2011; Golyaev et al. 2019) or mechanically inoculated JSBWMV from Bornum. CP-RT was amplified from leaves of the plants by RT-PCR. While PCR products from plants cultivated for three months in virus-containing soil contained wild type sized CP-RT, PCR products obtained from cDNA of the plants with prolonged cultivation after mechanical infection with

Table 1 Fluorescence lifetime values and percentage of FRET-FLIM between JSBWMV CP-RT:GFP and CP-RT:RFP

Expressed proteins	Localization	<i>N</i>	<i>n</i>	Fluorescence half life τ (ns)	SD	% FRET
CP-RT:GFP	dots	2	38	2,42	0,04	
CP-RT:GFP+CP-RT:RFP	dots	2	53	2,1	0,11	13,2
CP-RT:GFP	ER	2	229	2,43	0,05	
CP-RT:GFP+CP-RT:RFP	ER	2	162	2,05	0,08	15,6

dots dot-like structure, *ER* endoplasmic reticulum, *N* number of independent experiments, *n* number of measured points at the indicated localization, *SD* standard deviation. % FRET efficiency was calculated from the ratio of CP-RT:GFP τ values in the presence and absence of the RFP-tagged acceptor protein

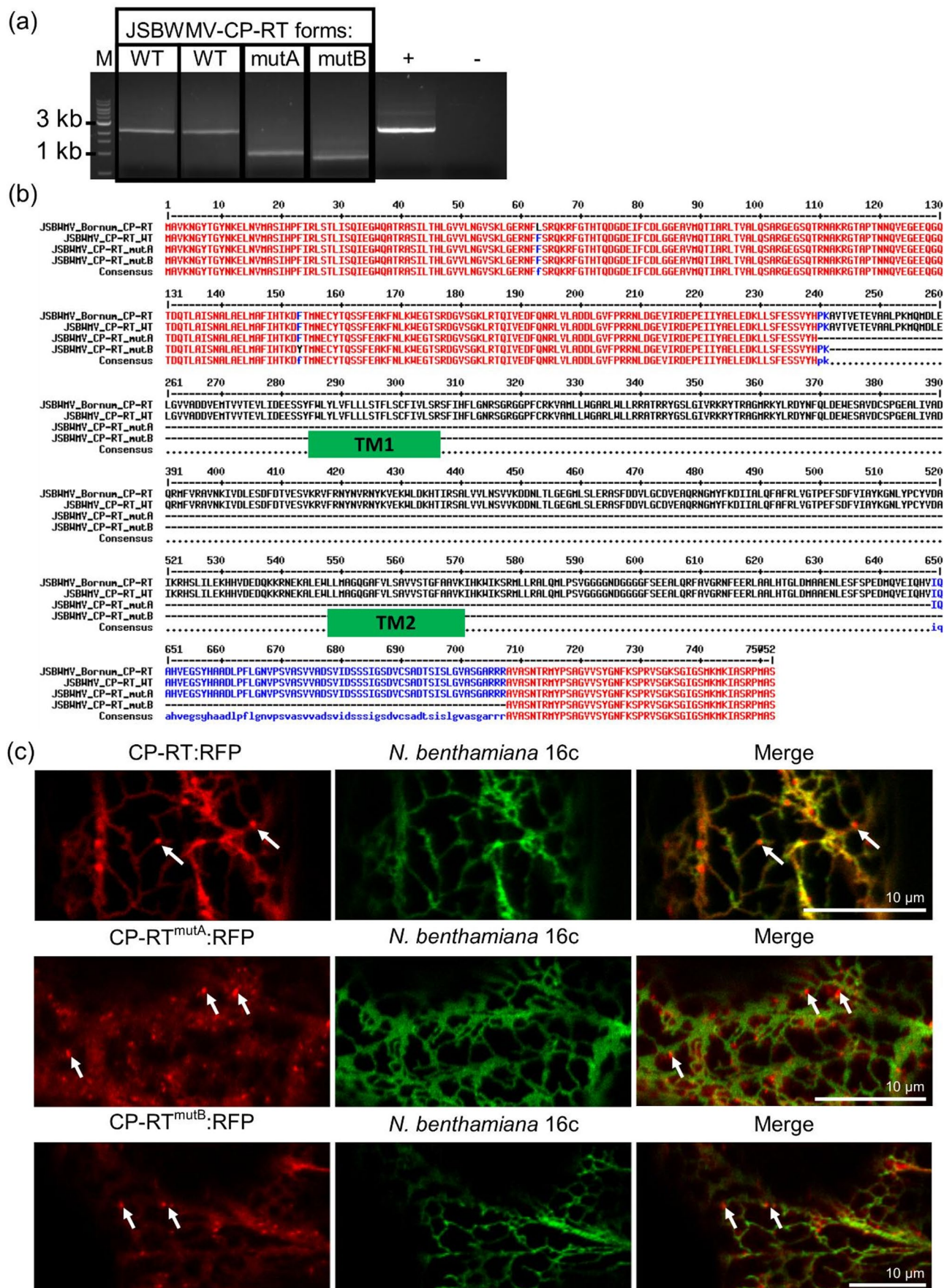


Fig. 5 Generation and characterization of CP-RT deletion mutants. (a) CP-RT was amplified by RT-PCR from RNA isolated from barley plants infected with the JSBWMV-Bornum isolate. WT, wild type CP-RT amplicon obtained by RT-PCR from plants grown from seeds in infected soil for three months. mutA and mutB, mutant CP-RT amplicon obtained from barley plants rub-inoculated with JSBWMV-Bornum and cultivated for six months (mutA) or one year (mutB), respectively, in the greenhouse. +, CP-RT amplicon generated from pJS2 cDNA. -, non-template control. M, 1 kb DNA ladder (NEB). (b) Amino acid sequence comparison between wild type (Rabenstein et al. 2011) and mutant CP-RT sequences. TM1 and TM2, predicted transmembrane domains. Red, sequence identity between the different alignments, blue, identical amino acids between three of the four sequences. Sequence alignment was created with MultAlin (Corpet 1988). (c) WT CP-RT:RFP (upper panels), CP-RT^{mutA}:RFP (Middle panels) and CP-RT^{mutB}:RFP (Lower panels) were expressed in *N. benthamiana* 16c plants via agroinfiltration. While WT CP-RT localized to the ER network and small, ER-associated particles, the mutants lost localization to the ER network and instead localized to the cytosol. Localization to the particles was maintained. Left panels, RFP channel; middle panels, GFP channel, right panels, overlay image of the RFP and GFP channels. Pictures were taken two days after agroinfiltration

primers complementary to the CP-RT 5' and 3' end were smaller compared to the wild type CP-RT (Fig. 5a). Moreover, sequencing of the PCR products revealed a mutation at nucleotide 187 (C → T), which resulted in a substitution of L63 → F63 compared to the published sequence for this isolate (Rabenstein et al. 2011 (NCIB: KU236377.1). Sequencing of the PCR products with smaller sizes revealed deletions between nucleotides 720–1947 (CP-RT^{mutA}) and between nucleotides 731–2129 (CP-RT^{mutB}). CP-RT^{mutB} contained a point mutation at nucleotide 458 (T → A), which resulted in a substitution of F153 → Y153 (Fig. 5b). The deletions comprised the two predicted TMs in the CP-RT sequence. Importantly, the 3' 129 nucleotides and open reading frame were maintained for both deletion mutants identified. Upon ectopic, agrobacterium-mediated expression in *N. benthamiana*, CP-RT^{mutA} and CP-RT^{mutB} localized to ER-associated dot-like structures similar to wild type CP-RT:GFP and CP-RT:RFP, but localization to the ER network was abolished (Fig. 5c). In addition, diffuse cytoplasmic localization was also observed.

Intact CP-RT protein appears to be involved in early infection stages

To investigate transmission of wild type and mutant CP-RT forms by the virus vector, *P. graminis*, as well as a role of the CP-RT forms in early infection events, we conducted hydroponic culture experiments with source plants, which were grown for different time periods in virus-containing soil and thus contained virus with different CP-RT forms (Fig. 6). Source root samples contained CP-RT with deletions in addition to wild type CP-RT (Fig. 6a and b) as indicated by RT-PCR of the CP-RT gene. Bait plants were introduced into the hydroponic culture (Fig. 6c) and the CP-RT forms

present in root and leaf samples of bait plants were determined. In roots and leaves of bait plants, wild type CP-RT was found to a greater extent compared to mutant forms of CP-RT at this time point (Fig. 6 d and e). These findings suggest a role for wild type CP-RT during transmission and/or early during infection. The fact that also mutant CP-RT forms could be found in bait plants may suggest a function of the shorter CP-RT protein during infection.

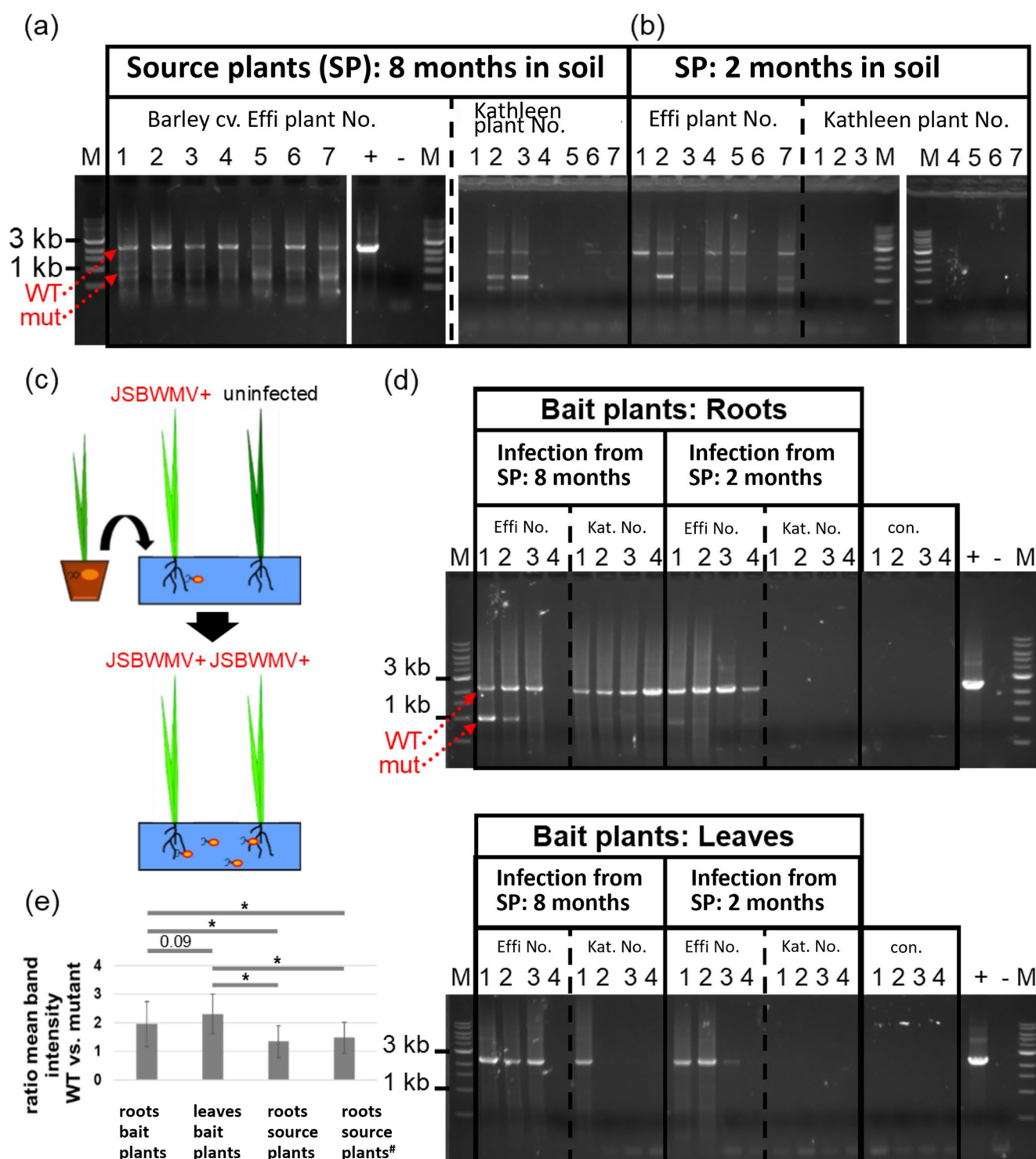
Discussion

In order to better understand how the JSBWMV CP-RT protein is involved in virus infection, we investigated subcellular localization, multimerization and transmission behavior of wild type and mutant forms of JSBWMV CP-RT.

CP-RT and the homolog proteins of plasmodiophorid-transmitted viruses share functions in virus transmission (Bragard et al. 2013; D'Ann Rochon et al. 2004). The CP-RT proteins of beny-, pomp-, furo- as well as the P2 protein of bymoviruses have been shown to contain TMs with conserved helices (Adams et al. 2001). A motif of four amino acids (KTER) in the TM domain has been shown to be required for benyvirus transmission by *P. betae* and *P. graminis* (Tamada et al. 1996; Lozano and Morales, 2009). A similar motif (VTER) has also been identified in wheat stripe mosaic virus, which occurs in association with *P. graminis* (Valente et al. 2019). The presence of the conserved TMs and motifs in these transmission proteins have led to the hypothesis that these structural features postulating membrane association are important for the transmission process.

We found that JSBWMV CP-RT localized to the ER network and small, ER-associated particles which were mobile along the ER network during infection. The idea of compartmentalized linked replication, defense suppression and movement crystallizes as one of the main models for regulated plant RNA virus distribution (Levy and Tilsner 2020). Transport of these viral replication compartments (VRCs) involves association of viral proteins and RNAs in complexes with ER membranes, other host membranes and cytoskeletal components (Jovanovic et al. 2023).

In the case of soil-borne viruses, association to the ER has been described for the CP-RT homologous P2 of wheat yellow mosaic virus (WYMV), which has been shown to rearrange the ER and to form replication-associated inclusion bodies (Sun et al. 2014). We found that fluorescent protein-tagged JSBWMV CP-RT localized to the ER network, however, no rearrangement of the ER into large inclusions, similar to those seen for WYMV P2, was observed during infection. Large, mainly perinuclear and smaller, mobile inclusions are known for potyvirus infection and are thought



to represent VRCs (Jovanovic et al. 2023; Xue et al. 2023). Interestingly, JSBWMV-CP-RT:GFP could be recruited to large ER-associated inclusions upon overexpression of ectopically expressed CP-RT:RFP probably through protein-protein interaction (Fig. S4). However, as large inclusions of fluorescent protein-labelled JSBWMV-CP-RT were not seen during infection, during which the CP-RT fusion

protein was expressed from the original viral promoter and the translational read-through codon was maintained, we presume that the small, mobile particles and the ER-network distribution of CP-RT represent the localization of functional forms of CP-RT. Interestingly, the punctate structures remained present for CP-RT mutants that lost the ability to associate with the ER network.

Fig. 6 CP-RT forms detected in source and bait plants in hydroponic transmission experiments. Barley cv. Effi and cv. Kathleen (Kat.) were used. **(a and b)** Agarose gel images showing CP-RT amplicons derived after PCR in source plants. Numbers indicate rows, in which amplicons from individual plants were resolved. Black boxes correspond to the different set ups. After eight or two months, respectively in JSBWMV-infected soil, source plants were removed from soil to insert them into hydroponic culture after washing the roots, sampling from individual roots and analyzing them for CP-RT forms by RT-PCR after RNA extraction. Apart from WT CP-RT amplicons with a size of approximately 2.5 kb, also smaller amplicons with sizes of approximately 1 kb or smaller were detected. After two months in soil, no infection of the tested Kathleen plants was observed. **(c)** Graphic illustration of the experimental set up. Plants grown in infected soil were carefully removed from the soil after eight or two months, respectively and roots washed carefully with tap water. The plants were pooled and introduced into hydroponic culture as source plants. Bait plants were germinated on tissue paper, introduced into the hydroponic culture and roots and leaves assayed for the presence of CP-RT forms five weeks after insertion of the bait plants. **(d)** Five weeks after insertion of the bait plants, bait plants were collected and the roots were rinsed under tap water. Subsequently, leaf and root samples were taken for RNA extraction. RT-PCR was performed to analyze CP-RT forms accumulating in the individual bait plants. Representative agarose gel images are shown. Right black box (con.) in d, set up in which no source plants were included. +, CP-RT amplicon generated from pJS2 plasmid cDNA. -, non-template control. M, 1Kb DNA ladder (NEB). The experiment was conducted three times, each time with 32 bait plants. **(e)** Quantification of band intensities of WT vs. mutant CP-RT forms. Relative quantification was made on agarose gels after RT-PCR. In each lane with a signal, the wild type CP-RT band area and the mutant CP-RT band area at approximately 1 kb (or an area around 1 kb in the absence of band signal) was encircled and mean grey values measured using ImageJ (Schindelin et al. 2012). Ratios between mean grey values for WT CP-RT area and mutant CP-RT area were calculated for each lane. The ratios were then averaged over all replicates and experiments and mean values $\pm$ SD calculated. Significant differences according to student's t-test $p < 0.001$ are shown by asterisks. The p-value between roots and leaves of bait plants was $p = 0.09$ (student's t-test). Bar 'roots source plants': As for source plants also a considerable number of CP-RT forms with sizes smaller than 1 kb were detected in addition to the band at approximately 1 kb, also these bands were quantified in relation to the WT CP-RT bands

For the furovirus Chinese wheat mosaic virus (CWMV), replicase was shown to localize to inclusions co-precipitating with cellular membranes (Yang et al. 2017) and the CWMV silencing suppressor localized to the ER (Sun et al. 2013b). Thus, these data may suggest that also furoviruses compartmentalize replication, defense suppression and movement in ER-associated mobile VRCs. Interestingly, CWMV MP has been reported to localize to ER-derived, mobile, vesicle-like structures resembling the dot-like particles observed here for CP-RT (Andika et al. 2013). Plasmodesmata targeting and the formation of small, mobile structures by CWMV MP depended on the secretory pathway. Also, JSBWMV infection appears to involve the secretory pathway, as JSBWMV CP-RT dot-like particles colocalized with particles labelled by the ERIS marker AtSYP72:RFP and with those labelled by Sar1-GTP: GFP, thus indicating that the particles mark specific sub-ER sites at the interface between ER

and Golgi. In support of this idea, BFA experiments indicated that reabsorption of the Golgi marker Man1:RFP into the ER initiates at CP-RT:GFP labeled sub-ER sites (Fig. 3). Interestingly, the tobacco etch virus (TEV) 6K2 protein colocalized with ER exit site (ERES) markers dependent of a functional secretory pathway (Wei and Wang 2008) and the P2-protein of the *P. graminis*-transmitted WYMV directly interacted with the COPII GTPase SAR1 (Sun et al. 2014). Moreover, 6K2 overexpression of potyvirus infection inhibited secretion through the secretory pathway and virus infection was reduced when COPI or COPII complex formation was impaired. The authors suggested that interference with the secretory pathway may assist in the development of ER-associated virus replication sites and support provision of biomolecules for virus replication.

With respect to the model of mobile VRCs for efficient virus replication and movement the association of CP-RT with the ER may represent a direct transport route for the virus to PD and between plant cells. Moreover, transport along the ER and membrane fusion events at the plasmodium membrane may allow transport between these organelles. By connecting virus particles or viral ribonucleoprotein (vRNP) complexes to transport routes along and between the plasmodium of the vector and host membranes, the CP-RT could fulfil its predicted function as virus-encoded transmission factor.

The integrity of the CP-RT protein or homologous proteins has previously been shown to be important for transmission by plasmodiophorid vectors (Tamada and Kusume 1991; Tamada et al. 1996; Reavy et al. 1998; Adams et al. 2001). Our findings from the hydroponic culture transmission experiments confirm these previous findings. While wild type and CP-RT mutant forms were detected in roots and to a lesser extent in leaves of newly infected bait plants in hydroponic culture, wild type CP-RT forms were present in higher abundance in bait vs. source plants. No plant contained only mutant CP-RT forms. This may suggest that wild type CP-RT may be important during early stages of infection, while mutant CP-RT may play a role at later infection stages. Interestingly, localization of CP-RT to the ER was lost in naturally evolved mutants in the CP-RT protein, suggesting that the localization to the ER network is important for CP-RT function in virus transmission and during early infection. Future studies will address the molecular mechanisms of transmission, replication and movement and the involvement of the ER and secretory pathway in this process. For PMTV it was shown that CP-RT was needed for movement (Chapman et al. 2008). PMTV systemic movement of the viral RNA encoding CP and CP-RT proteins was inhibited if only CP or truncated CP-RT was expressed (Torrance et al. 2009), thus suggesting a role for the C-terminal domain of CP-RT in movement. Taken together, our data highlight the critical role of ER localization in virus infection.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s00709-026-02163-4>.

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Author contributions AN conceived the experiments; NS, AN designed and planned the experiments; NS, CS, PB and EB conducted the experiments; NS, CS, EB, and AN analyzed the data; AN and NS wrote the manuscript with contributions of CS, and EB. All authors approved the final version of the manuscript.

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Declarations

Accession numbers The TAIR accession number of the cloned Arabidopsis *AtSYP72* gene is AT3G45280. The GenBank accession numbers for JSBWMV strain JT are NC_038850.1 (RNA1) and NC_038851.1 (RNA2). Genbank accession numbers for JSBWMV-Bornum are MN123252.1 (RNA1) and KU236377.1 (RNA2).

Conflicts of interest: The authors declare no conflict of interest.

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