Immunodetection and fluorescent microscopy of transgenically expressed hordeivirus TGBp3 movement protein reveals its association with endoplasmic reticulum elements in close proximity to plasmodesmata

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The subcellular localization of the hydrophobic TGBp3 protein of Poa semilatent virus (PSLV, genus Hordeivirus) was studied in transgenic plants using fluorescent microscopy to detect green fluorescent protein (GFP)-tagged protein and immunodetection with monoclonal antibodies (mAbs) raised against the GFP-based fusion expressed in E. coli. In Western blot analysis, mAbs efficiently recognized the wild-type and GFP-fused PSLV TGBp3 proteins expressed in transgenic Nicotiana benthamiana, but failed to detect TGBp3 in hordeivirus-infected plants. It was found that PSLV TGBp3 and GFP–TGBp3 had a tendency to form large protein complexes of an unknown nature. Fractionation studies revealed that TGBp3 represented an integral membrane protein and probably co-localized with an endoplasmic reticulum-derived domain. Microscopy of epidermal cells in transgenic plants demonstrated that GFP–TGBp3 localized to cell wall-associated punctate bodies, which often formed pairs of opposing discrete structures that co-localized with callose, indicating their association with the plasmodesmata-enriched cell wall fields. After mannitol-induced plasmolysis of the leaf epidermal cells in the transgenic plants, TGBp3 appeared within the cytoplasm and not at cell walls. Although TGBp3-induced bodies were normally static, most of them became motile after plasmolysis and displayed stochastic motion in the cytoplasm.

INTRODUCTION

The positive-stranded RNA genome of Poa semilatent virus (PSLV, genus Hordeivirus) consists of three RNAs, termed α, β and γ. RNAs α and γ of hordeiviruses are essential for virus genome replication and encode two viral replicate components and a regulatory protein, whereas RNA β encodes the coat protein and three proteins involved in virus movement (Lawrence & Jackson, 1999; Lawrence et al., 2000; Savenkov et al., 1998; Solovyev et al., 1996). Genes of the hordeivirus movement proteins (MPs) are organized in the evolutionarily conserved gene module called the ‘triple gene block’ (TGB), which is also found in some other plant viruses of the proposed families Tubiviridae and Potexviridae (Herzog et al., 1998; Koenig et al., 1998; Lawrence & Jackson, 2001a; Lough et al., 1998; Morozov & Solovyev, 1999; Morozov et al., 1989). All three TGB-encoded proteins, referred to as TGBp1, TGBp2 and TGBp3 according to their position, are necessary for cell-to-cell movement of virus (Beck et al., 1991; Gilmer et al., 1992; Herzog et al., 1998; Krishnamurthy et al., 2002; Petty & Jackson, 1990).

Cell-to-cell movement of plant RNA viruses is generally thought to involve specific ribonucleoproteins (RNPs) composed of virus RNA and proteins (reviewed in Carrington et al., 1996; Citovsky & Zambrzyski, 1991; Haywood et al., 2002). In hordeiviruses, and presumably in other TGB-containing viruses of the proposed family Tubiviridae...
(genera * Hordeivirus, Pecluvirus, Pomovirus* and *Benyvirus*), the movement-related RNPs are formed by the TGBp1 protein, which has RNA-binding properties and an RNA helicase activity in vitro (Bleykasten et al., 1996; Brakke et al., 1988; Cowan et al., 2002; Donald et al., 1997; Kalinina et al., 2001; 2002). The two other TGB products of hordei- and potexviruses, TGBp2 and TGBp3, are highly hydrophobic and represent integral membrane proteins (Morozov et al., 1989, 1990, 1991; Solovyev et al., 1996, 2000).

In plant cells transiently expressing TGBp2 protein, it is associated mostly with the endomembrane system, particularly the tubular network of cortical endoplasmic reticulum (ER) and Golgi-like structures (Cowan et al., 2002; Solovyev et al., 2000). In a transient expression assay, TGBp2 of *Potato virus X* (PVX) was able to modify the size-exclusion limit of plasmodesmata (Tamai & Meshi, 2001). Moreover, TGBp2 and TGBp3 of PVX may even traffic from cell to cell in some host plants (Krishnamurthy et al., 2002). TGBp3 was localized to cell wall-associated membrane bodies (Cowan et al., 2002; Solovyev et al., 2000; Zamyatin et al., 2002). Importantly, TGBp3 was able to change the localization of TGBp2, targeting this protein to TGBp3-containing peripheral bodies. The TGBp3-directed subcellular targeting seems not to involve sequence-specific interaction of the TGBp2 and TGBp3 molecules and can also occur in mammalian cells, suggesting involvement of an universal mechanism for membrane protein transport (Solovyev et al., 2000; Zamyatin et al., 2002).

In hordeviruses and other TGB-containing viruses of the family *Tubiviridae*, TGBp2 and TGBp3 specifically target TGBp1, and presumably TGBp1-formed movement-competent RNPs, to plasmodesmata-associated sites (Erhardt et al., 1999; 2000; Lawrence & Jackson, 2001b). This intracellular transport of TGBp1 is believed to be driven by the TGBp3 subcellular targeting signal (Solovyev et al., 2000). However, the mode of TGBp3 functioning is still obscure and questions remain concerning the molecular mechanisms of protein subcellular sorting to peripheral cell compartments directed by TGBp3 and possible additional movement-related TGBp3 functions.

Attempts at TGBp3 immunodetection in plants infected with the type hordeivirus, *Barley stripe mosaic virus* (BSMV), have failed (Donald et al., 1993). The lack of an immunodetection system has impeded characterization of TGBp3 synthesized during infection and complicated studies of its possible interactions with host protein and lipid components and its compartmentalization. In this paper, we have used subcellular fractionation, immunodetection and confocal microscopy to localize the PSLV TGBp3 protein in transgenic *Nicotiana benthamiana* expressing the wild-type and GFP-fused protein.

**METHODS**

**Construction of recombinant clones.** For expression of GFP, the 18 kDa PSLV TGBp3 protein (18K) or its translational fusion with GFP gene (GFP–18K) in *E. coli* we used vector pQE30N, which is a derivative of pQE30 (Qiagen) with an Ncol site introduced into the plasmid polylinker (Solovyev et al., 1999). The 18K gene was excised from plasmid pRT-GFP-18K (Solovyev et al., 2000) with Ncol and Xhol and GFP–18K was excised from pRT-GFP-18K by partial digestion with Ncol and complete digestion with Xbal. The genes were then cloned into similarly digested pQE30N. The GFP gene was isolated as an Ncol–Xbal fragment from pRT-GFP-S65T (Solovyev et al., 2000) and ligated into Ncol/Xbal-digested pQE30N (Fig. 1).

The two hydrophilic regions of PSLV TGBp3 were expressed in *E. coli* as fusions with mouse dihydrofolate reductase (DHFR). The 5’–terminal region of the PSLV TGBp3 gene encoding a hydrophilic segment located at aa 1–61 and the central region encoded aa 82–139 were amplified by PCR using pQE-GFP-18K as the template. Both PCR products were digested with *Bam*HI and Xbal and cloned into similarly digested pQE40 (Qiagen). The expression vectors carrying the sequences of the N-terminal and central hydrophilic regions of TGBp3 were designated pQE-DHFR-111 and pQE-DHFR-35, respectively (Fig. 1).

BSMV mutant P684 containing the PSLV TGB and PVX.GFP, the PVX derivative expressing GFP, have been described previously (Fedorkin et al., 2001; Solovyev et al., 1999).

**Expression of cloned genes in *E. coli* and protein purification.** Recombinant pQE-based expression vectors were transformed into *E. coli* strain M15[pREP4] (Qiagen). Induction of protein expression in the bacteria carrying vectors for GFP- and DHFR-fused polypeptides was carried out essentially as described previously (Solovyev et al., 1999). Purification of the proteins on Ni-NTA resin (Qiagen) was according to manufacturer’s instructions. Before immunization of mice, the protein was dialysed against 0.5% SDS.

**Monoclonal antibodies and PEPSCAN analysis.** Four 8-week old BALB/c mice were each immunized intraperitoneally with protein suspension in water containing 0.5% SDS (50–60 µg PSLV GFP–18K) and boosted twice with the same dose at 2-week intervals. A final boost was given by the intraperitoneal route with 50 µg of the antigen without Freund’s adjuvant. Three days later, the splenocytes were harvested and fused with the mouse plasmacytoma cell line Sp2/0 using 45% PEG 1500 (Erokhina et al., 2000). Hybridomas secreting the specific monoclonal antibodies (mAbs) were identified by indirect ELISA. These hybridomas were cloned twice under conditions of limiting dilution.

Overlapping sets of synthetic octapeptides, progressively moving along by two residues and covering the 20 C-terminal residues of GFP plus aa 1–58 (N-terminal hydrophylic segment) and the aa 81–143 (central hydrophylic segment) of PSLV TGBp3, were prepared on derivatized cellulose sheets (SPOTs kit; Genosys) using activated F-moc amino acid residues, as described (Erokhina et al., 2001).

**Production of transgenic plants expressing PSLV TGBp3 and its GFP fusion protein.** Transgenic plants expressing the 18K protein or its translational fusion with the GFP gene (GFP–18K) were generated with the binary vectors pBin-18K and pBin-GFP-18K, which carried 35S promoter expression cassettes from the plasmids pRT–18K and pRT-GFP-18K, respectively (Solovyev et al., 2000). *N. benthamiana* leaf discs were transformed with *Agrobacterium tumefaciens* AGL carrying vectors pBin-18K and pBin-GFP-18K and transformants were regenerated on a kanamycin-containing selective medium. *R₀* plants were self-pollinated and *R₁* progeny were analysed by Northern blotting for the presence of 18K-specific mRNAs (Fig. 2).

**Subcellular fractionation and Western blot analysis.** Leaf tissue (100 mg) was ground into a fine powder with liquid nitrogen in 2 ml buffer A (400 mM sucrose, 100 mM Tris/HCl, 10 mM KCl, 0.5 mM EDTA, 1 mM DTT; pH 7.6) and 0.1 ml of buffer B (400 mM sucrose, 100 mM Tris/HCl, 10 mM KCl, 0.5 mM EDTA, 1 mM DTT; pH 7.6) was added. The homogenate was homogenized with a Dounce homogenizer and centrifuged at 12000 × g for 30 min. The supernatant was centrifuged at 100000 × g for 60 min. The pellet was suspended in 0.8 ml buffer C (400 mM sucrose, 100 mM Tris/HCl, 10 mM KCl, 0.5 mM EDTA, 1 mM DTT; pH 7.6) and homogenized with a Dounce homogenizer. The homogenate was centrifuged at 100000 × g for 60 min. The supernatant was labelled as soluble fraction. The pellet was extracted by buffer C containing 20% Triton X-100, 200 mM NaCl, 5 mM CaCl₂, 100 mM Tris/HCl, pH 7.6. The homogenate was homogenized with a Dounce homogenizer and centrifuged at 100000 × g for 60 min. The pellet was suspended in 2 ml buffer C and homogenized with a Dounce homogenizer. The homogenate was centrifuged at 100000 × g for 60 min.

**RESULTS**

**Construction of recombinant TGBp3 clones.** Total RNA was extracted from 18-week-old leaves of *N. benthamiana* expressing the wild-type and GFP-fused TGBp3 proteins. Total RNA was subjected to Northern blotting and hybridized with a digoxigenin-labelled probe. A hybridization signal was observed in the 18K region (Fig. 9).

**Western blot analysis.** Leaf samples were extracted in buffer A containing 1% SDS. Total protein was subjected to SDS-PAGE under reducing conditions with 18% acrylamide. Proteins were transferred onto a nitrocellulose membrane and probed with a polyclonal antibody raised against the 18K protein and a monoclonal antibody directed against GFP. A hybridization signal was observed in the 18K region (Fig. 10).
5 mM MgCl₂, 10 mM β-mercaptoethanol, 1 mM PMSF, pH 7.5). The slurry was filtered through one layer of Miracloth and the filtrate was either centrifuged at 30,000 g for 30 min to yield a pellet of combined P1 plus P30 fraction (MEM fraction) enriched in organelles and miscellaneous membranes and S30 fraction (soluble protein fraction), or centrifuged for 10 min at 1000 g to yield a pellet fraction (P1) enriched in nuclei. In the latter case, the supernatant was centrifuged at 3000 g for 10 min to obtain pellet fraction P3 composed of chloroplast fragments and mitochondria. The resulting supernatant (S3) was centrifuged at 30,000 g for 30 min to give a P30 fraction enriched in cell membranes. The Miracloth residue was washed in buffer A containing 2% Triton X-100 followed by centrifugation (10,000 g) and resuspension in buffer A and this cycle was repeated until the pellet became white to yield the cell wall (CW) fraction. All fractions were resuspended in 1/56 Laemmli sample buffer containing either 9 M urea or as described in the figure legends. For further subcellular fractionation on sucrose gradients, the S3 fraction from transgenic plants (~600 μl) was loaded on a 12 ml linear 25–55% (w/w) sucrose gradient made in the extraction buffer, which contained either 5 mM or 0.1 mM magnesium chloride. Immunoblot analysis was performed using mAbs against the PSLV GFP–18K fusion protein or polyclonal antiserum raised against the intrinsic ER protein AtSEC12 (Bar-Peled & Raikhel, 1997) from Arabidopsis thaliana (RoseBiotech).

Unfractionated leaf extracts (TE preparation) were prepared by adding 200 μl 1/5× Laemmli sample buffer, containing either 9 M urea or as described in the figure legends, to 100 mg of plant tissue ground to a fine powder with liquid nitrogen.

Fractionated proteins were transferred from 8–12% gradient SDS-polyacrylamide gels to nitrocellulose membrane (Immobilon-P). Reactions were revealed with the enhanced chemiluminescence immunoblotting system (Amersham).

**Fluorescent microscopy.** Fluorescence was detected using a Zeiss Axioscope 20 fluorescence microscope, as previously described (Zamyatnin et al., 2002). Callose was visualized in leaf cells with 0.1% (w/v) sirofluor-containing aniline blue (Merck) in 1 M glycine, pH 9.5, as previously described (Smith & McCully, 1978; Stone et al., 1984). To induce plasmolysis, leaf pieces were incubated in 0.5 M mannitol solution for 5 min. Particle bombardment of N. benthamiana leaves was performed using the flying disc method with a high-pressure helium-based PDS-1000 system (Bio-Rad), as described by Morozov et al. (1997).
RESULTS

Monoclonal antibodies and epitope mapping

Using the expression vector pQE-GFP-18K (Fig. 1), the 6-His-tagged GFP-fused TGBp3 of PSLV was expressed in E. coli and affinity-purified. Immunization of mice with the 6-His-GFP–18K fusion resulted, after cloning, in 26 mAb-producing hybrid clones, which were tested by indirect ELISA and immunoblots with purified GFP and two recombinant proteins, DHFR-111 and DHFR-35, representing DHFR fusions of the N-terminal and central hydrophilic regions of the PSLV TGBp3, respectively (Fig. 1). Four mAbs (3B4, 3B6, 3B9 and 3B12) gave a strong positive reaction with GFP but not with DHFR-111 or DHFR-35 and three clones (mAbs 2D3, 2D4 and 2D5) gave a strong reaction with GFP–18K and weaker signals with both GFP and DHFR-111, whereas mAb 1D4 bound only to DHFR-35 (data not shown).

Two mAbs, 1D4 and 2D5, showing different specificity to GFP, DHFR-111 and DHFR-35, were selected for peptide scanning analysis. Two overlapping sets of synthetic peptides were used. To locate the epitope of mAb 2D5, a peptide set was taken spanning the GFP–18K fusion protein region, which included the 20 C-terminal residues of GFP and the 58 N-terminal residues of PSLV TGBp3. This mAb strongly reacted with the peptide sequence LELYKGSMA located at the junction of GFP (the six C-terminal GFP residues are shown in bold) and the N-terminal hydrophilic segment of the PSLV 18K protein. The C-terminal half of this octapeptide was identical to the DHFR-111 sequence at the junction of DHFR and PSLV TGBp3. This could explain the reaction of mAb 2D5 with DHFR-111 in addition to GFP and GFP–18K (Figs 1 and 3, and data not shown). To locate the epitope of mAb 1D4, a peptide set was used that spanned aa 81–143 of PSLV TGBp3. The mAb 1D4 strongly reacted with the peptide sequence YVKGGY positioned in the central hydrophilic region (aa 87–92) just downstream of the first hydrophobic stretch of PSLV TGBp3 (data not shown).

Transgenic plants expressing the PSLV TGBp3 and its GFP fusion protein

In both the pBin-18K and pBin-GFP-18K transformants, single bands of the expected sizes, approximately 0.5 kb for the 18K-transgenic plants and 1.3 kb for the GFP–18K-transgenic plants, were detected by Northern blot in several plant lines (Fig. 2). Analysis of these lines by Western blotting with monoclonal antibodies to PSLV 18K demonstrated expression of proteins of the expected sizes, either an 18 kDa protein in 18K-transgenic lines, or a 45 kDa protein in the GFP–18K-transgenic lines (Fig. 3A; Fig. 4). Based on these analyses, four transgenic lines were selected for further work. Lines R3 and R15 were used for the 18K transgene and lines R1 and R5 for GFP–18K transgene (Fig. 2). For each transgene, similar results were obtained for the two lines. Therefore, only data for lines R1 and R15 are presented below.

Immunodetection of PSLV TGBp3 in transgenic and infected plants

Initially, we analysed N. benthamiana plants infected with the chimeric BSMV/PSLV virus P684, which carries the PSLV
TGB region in place of that of BSMV (Solovyev et al., 1999). For sample preparation, inoculated leaves were first taken at 5 days post-inoculation (p.i.) when the chlorotic symptoms appeared. Subsequently, inoculated and systemically infected leaves were harvested at later steps of infection. Samples were fractionated to yield the MEM (P1 plus P30), S30 and CW fractions. Neither mAb 2D5 nor 1D4 was able to detect the PSLV TGBp3 protein in any subcellular fraction at any infection stage tested, even when a sensitive enhanced chemiluminescent detection system was used (Fig. 3A).

To test whether mAbs 2D5 and 1D4 were able to recognize GFP–18K expressed in plants, the leaves of transgenic N. benthamiana plants were analysed by Western blotting. As a control, PVX.GFP-infected N. benthamiana was used. It was found that both mAbs 2D5 and 1D4 readily detected GFP–18K in the MEM and CW fractions (Fig. 3A and 4). However, the protein did not appear in the soluble protein (S30) fraction (Fig. 3A). In the control, as expected, GFP was readily detected in extracts from PVX.GFP-infected plants probed with mAb 2D5 but not with mAb 1D4 (Fig. 3A).

Importantly, substantial amounts of GFP–18K (Fig. 3) and 18K (Fig. 4) were found in the form of high molecular mass bands with molecular masses greater than 175 kDa. These aggregates were stable following β-mercaptoethanol treatment and boiling but dissociated in 9 M urea (Fig. 3B).

**PSLV TGBp3 is an integral membrane protein associated with the ER**

To determine whether the PSLV TGBp3 and its complexes were peripherally associated with or embedded into membranes, the P30 fraction was subjected to different biochemical treatments. The results showed that TGBp3 could not be extracted from the P30 pellet by treatment with 0.1 M Na2CO3, 1 M NaCl or 2% Triton X-100. The position of the protein in the gel is indicated.

Previously, we have demonstrated that PSLV TGBp3 protein-induced peripheral membrane bodies contained a resident ER fluorescent protein, suggesting that these bodies could originate from an ER compartment (Zamyatnin et al., 2002). To verify the origin of the peripheral bodies biochemically, the P3 fraction of the GFP–18K transgenic plants was fractionated on a 20–55% sucrose gradient (Fig. 5). As a molecular marker, we used antibodies to the ER-specific membrane protein AtSEC12 (Bar-Peled & Raikhel, 1997) from A. thaliana. If subcellular fractionation was performed in the presence of 0.1 mM Mg2+, the GFP–18K protein and its high molecular mass aggregates were found in a number of fractions forming a distinct peak (Fig. 5, lower panel, fractions 11–14), which largely coincided with the AtSEC12-containing peak (Fig. 5, lower panel, fractions 10–13). On the other hand, the plasma membrane-specific aquaporin PIP1b was found mostly in other gradient fractions (data not shown). These observations showed that GFP–18K is mainly

**Fig. 4.** Extraction of the 18K protein from the P30 pellet with 0·1 M Na2CO3, 1 M NaCl or 2% Triton X-100. The position of the protein in the gel is indicated.

**Fig. 5.** Western blot analysis of fractions from a sucrose density gradient. Membrane preparations with 5 mM MgCl2 (+Mg2+) or 0·1 M MgCl2 (−Mg2+) from transgenic plants expressing GFP–18K were separated on a 25–55% sucrose gradient. Fractions were analysed with antibodies to 18K (mAb 1D4) and to the ER-specific protein AtSEC12, as indicated on the left. Fraction numbers are indicated above each lane. Sedimentation was from left to right.
associated with the ER membranes. If subcellular fractionation was performed in the presence of 5 mM Mg\(^{2+}\), there was a slight shift of the peak compared with 0-1 mM Mg\(^{2+}\) and the appearance of a minor peak for both GFP–18K and AtSEC12 (Fig. 5, upper panel, fractions 17–18). It is known that low Mg\(^{2+}\) concentration releases ribosomes attached to the ER, and a change of membrane mobility has also been observed in other studies using sucrose-gradient fractionation of the plant ER in the presence or absence of EDTA (for example, see Pedrazzini et al., 1997). Moreover, membrane ER proteins used as markers for the sucrose-gradient fractionation of plant and yeast endomembranes often show minor peaks of ER-containing fractions (for example, see Ballensiefen et al., 1998; Sterling et al., 2001).

Non-fused TGBp3 from transgenic plants behaved similarly to GFP–18K in sucrose gradients (data not shown). These data suggested that transgenically expressed PSLV TGBp3 is bound to membranes originating from the ER.

Fluorescent microscopy of GFP–18K expressed in transgenic plants

The GFP–18K-expressing plants were examined using confocal laser scanning microscopy. In single optical sections traversing the middle of the epidermal cell layer, the GFP fluorescence was found in cell wall-associated bodies (Fig. 6B), similar to that observed previously in cells expressing the GFP–18K protein (Solovyev et al., 2000; Zamyatnin et al., 2002). In projections of a series of optical sections, small GFP–18K-containing bodies were also visible on the upper and lower cell surfaces (Fig. 6A), also consistent with the transient expression data (Solovyev et al., 2000). Therefore, the distribution of GFP–18K in transgenic...
N. benthamiana plants was similar to that found in particle bombardment experiments. However, large cell-wall-associated bodies typically found in cells with high levels of transiently expressed GFP–18K were not found in the GFP–18K-transgenic plants. This observation is in agreement with data showing that the size of the peripheral bodies correlates with TGBp3 expression levels (Zamyatnin et al., 2002).

Examination of GFP–18K-expressing epidermal cells of the transgenic plants under higher magnification revealed that the peripheral fluorescent bodies located at the cell wall represented twin structures consisting of pairs of disconnected bodies located on opposite sides of the cell wall (Fig. 6C). Therefore, when the protein is expressed in the transgenic plant cells, the peripheral bodies formed in two neighbouring cells localized just opposite one another. Similar twin bodies were found in plant tissues infected with a tobacco mosaic virus vector expressing a GFP-fused TGBp3 protein of Potato mop-top virus (PMTV) (Cowan et al., 2002). The structural basis for formation of the twin bodies could be provided by plasmodesmata, assuming association of TGBp3-containing bodies with opposite neck regions of plasmodesmata in the same pit field (Blackman & Overall, 2001; Cowan et al., 2002; Erhardt et al., 2000; Lawrence & Jackson, 2001b).

TGBp3 is localized to cytoplasmic structures close to plasmodesmata

To verify the hypothesis that the GFP–18K-containing peripheral bodies were associated with plasmodesmata, we attempted to visualize plasmodesmata-associated callose with sirofluor, a specific fluorescent dye (Smith & McCully, 1978; Stone et al., 1984). Fluorescent microscopy of non-transgenic control plants treated with sirofluor-containing aniline blue demonstrated staining of discrete structures at cell walls. At higher magnifications, these structures sometimes appeared as twin bodies located at opposite sides of the cell wall (Fig. 6D), which was similar to previously described callose staining (Oparka et al., 1997). Phenotypically, the plasmodesmata-associated callose depositions visualized by the sirofluor staining were strikingly similar to the GFP–18K-containing peripheral bodies found in transgenic plants (Fig. 6C).

As sirofluor has an emission maximum similar to that of GFP, their fluorescence cannot be easily distinguished. Therefore, in experiments with sirofluor staining we used DsRed–18K – the 18K protein tagged with DsRed, a red fluorescent protein isolated from Discosoma sp. As described previously, localization of DsRed–18K is similar to that of GFP–18K (Zamyatnin et al., 2002). To compare the subcellular localization of DsRed–18K and callose, the leaves of N. benthamiana were bombarded with prT-DsRed-18K and then treated with sirofluor-containing aniline blue solution. Using epifluorescence microscopy, DsRed–18K-containing peripheral bodies were located in the vicinity of the plasmodesmata-related sirofluor-stained callose depositions (Fig. 6F–H). Interestingly, not all the plasmodesmata-related structures visualized by this treatment were associated with DsRed–18K-containing peripheral bodies. Moreover, different sirofluor-stained structures were similar in size to each other, whereas the size of the 18K-containing peripheral bodies varied considerably (Fig. 6F–H and data not shown).

To distinguish between binding of the TGBp3-containing peripheral bodies to the cell wall, plasma membrane or cortical cytoplasm elements, the leaf portions of transgenic N. benthamiana plants expressing GFP–18K were subjected to plasmolysis by mannitol treatment and epidermal cells were observed under the microscope. After the treatment, gaps between cell walls and plasma membranes were visible in the bright field (Fig. 6E). Fluorescent microscopy revealed that, after plasmolysis, GFP–18K-containing bodies appeared within the cytoplasm but not cell walls (Fig. 6E, arrows). Importantl, the GFP–18K-containing bodies, which are normally static in the cells of transgenic plants, became mobile after plasmolysis and displayed random motion in the cytoplasm (data not shown).

DISCUSSION

Earlier microscopic studies of fluorescent protein fusions with TGBp3 transiently expressed in bombarded epidermal leaf cells provided evidence for TGBp3 association with specific membrane bodies near the cell wall (Cowan et al., 2002; Solovyev et al., 2002; Zamyatnin et al., 2002). In this paper, to characterize further the subcellular association of TGBp3, we have conducted biochemical analysis of subcellular fractions and fluorescent microscopy of transgenic N. benthamiana plants expressing the PSLV TGBp3 or its fusion with GFP.

Although TGBp3 of PVX was detected by immunological methods in infected plants as a component of the CW fraction (Hefferon et al., 1997), immunodetection of TGBp3 in hordei- and benyvirus infections failed (Donald et al., 1993; Niesbach-Klösgen et al., 1990). In this paper, we were also unable to detect the presence of PSLV TGBp3 in virus-infected plants (from 5 to 18 days p.i.) with sensitive mAbs combined with an enhanced chemiluminescence immunoblotting system (Fig. 3). Most probably, TGBp3 is expressed in amounts too low to be detected in infected leaf tissues. Indeed, it was found that, in BSMV-infected protoplasts, TGBp3 was expressed at a tenfold lower level compared with TGBp2 (Zhou & Jackson, 1996).

Immunodetection of the PMTV GFP–TGBp3 expressed from a tobacco mosaic virus-based vector has demonstrated its association with the CW and membrane fractions (P1 and P30) (Cowan et al., 2002). In agreement with these data, it was found that PSLV GFP–18K (Fig. 3A) and 18K (Fig. 4 and data not shown) in transgenic plants were exclusively associated with the MEM (P1 and P30) and CW fractions. Complementation studies using these transgenic plants argue in favour of functional equivalence of transgenically expressed GFP–18K and 18K. In both types of transgenic plants, cell-to-cell and systemic movement of BSMV mutant...
P684 containing the PSLV TGB was inhibited (data not shown) in accordance with findings that independent expression of TGBp3 results in blockage of TGB-mediated virus cell-to-cell movement (Bleykasten-Grosshans et al., 1997). Moreover, despite the lack of complementation of P684 with disabled TGBp3 gene, both types of transgenic plants efficiently complemented cell-to-cell and long-distance movement of PVX with disabled TGBp3 gene (data not shown).

An unusual property of 18K and GFP–18K is the ability to form high molecular mass aggregates in SDS-PAGE with 4–6 M urea, dissociating in 9–12 M urea (Figs 3 and 4). These protein bands may correspond to natural TGBp3 forms and represent homomultimeric protein complexes or aggregates with host components. Indeed, TGBp3 was recently found to self-interact and thus, has the potential to form homomultimers (Cowan et al., 2002). However, integral membrane proteins may form high molecular mass aggregates that are artefacts of the protein boiling procedure in a standard Laemmli buffer. The SDS-resistant thermoaggregation is not observed when SDS/HEPES buffer and dithiothreitol are used instead of Tris and mercaptoethanol in the gel loading buffer (Sagne et al., 1996). However, such replacement had no obvious effect on the TGBp3 aggregates (data not shown). Additionally, SDS-resistant thermoaggregation results in the almost complete disappearance of a monomeric protein band with a concomitant accumulation of aggregates (Sagne et al., 1996), which is not the case with TGBp3 (Figs 3 and 4). Therefore, it is likely that PSLV TGBp3 expression in transgenic plants resulted in the formation of specific homomultimeric protein complexes. Recently, the high molecular mass aggregates were also detected in plants expressing PMTV GFP–TGBp3 fusion protein (Cowan et al., 2002).

When an isolated P30 fraction from PSLV TGBp3-expressing transgenic plants was treated with agents that are known to solubilize peripheral membrane proteins, TGBp3 remained associated with the pellet (Fig. 3B). Moreover, the strong anionic detergent SDS and the non-ionic detergent Triton X-100, known to efficiently solubilize integral membrane MPs (Huang & Zhang, 1999; Reichel & Beachy, 1998), were not sufficient for extraction of the bulk of the PSLV TGBp3 from the P30 fraction (Fig. 3B and data not shown), suggesting that it is not only hydrophobic interactions with cell membranes that govern TGBp3 binding with the rough membrane fraction.

To characterize the potential association of the TGBp3-induced bodies with cell membranes, sucrose-gradient fractionation of the cell membranes from transgenic plants was performed (Fig. 5). Immunodetection of the membrane-embedded resident ER protein AtSEC12 (Bar-Peled & Raikhel, 1997) showed that, as expected from microscopic data (Zamyatin et al., 2002), PSLV TGBp3 accumulated in the ER-derived membrane fraction (Fig. 5).

The regular distribution of fluorescent opposing bodies in transgenic plants expressing GFP–18K (Fig. 6C) suggested that TGBp3 might be incorporated into membrane compartments near the plasmodesmata orifice (plasmodesmata neck region). Our data on the co-localization of callose deposits and TGBp3-induced bodies strongly support this hypothesis (Fig. 6F–H) (see Botha & Cross, 2000; Blackman & Overall, 2001; Cowan et al., 2002). Fluorescent microscopy of N. benthamiana expressing GFP–18K revealed that the peripheral bodies are pulled away from the plant cell wall together with the plasma membrane during mannitol-induced plasmolysis (Fig. 6E). Concomitantly, the TGBp3-containing fluorescent bodies in plasmolized cells became motile, displaying stochastic motions in the cytoplasm (data not shown). This is in contrast to the static behaviour of the peripheral bodies in normal epidermal cells of bombarded and transgenic plants that express GFP–TGBp3 (data not shown and Solovyev et al., 2000). It was found that a membrane sucrose synthase that is bound to the plasma membrane and cell wall remains wall-associated after plasmolysis (Amor et al., 1995). In contrast, our finding that the GFP–18K-containing bodies became motile suggested that they may be physically associated with the desmotubule, which can be damaged or even torn after plasmolysis (Tilney et al., 1991), rather than with the cell wall or plasma membrane.

Taking into account the localization of TGBp3 to plasmodesmata and its possible association with the desmotubule, a mechanism for TGBp3-induced intracellular protein transport in virus infection can be hypothesized. At the sites of their synthesis in the ER membrane, the TGBp2 and TGBp3 proteins might form specific compartments, which are then transported using the targeting signal of TGBp3 to a specific plasmodesmal receptor by diffusion along the ER network (without exiting the ER), similar to lipids found to be able to transverse plasmodesmata (Cantrill et al., 1999; Grabski et al., 1993; Zamyatin et al., 2002). Alternatively, TGBp3 may form specific ER-derived membrane containers (vesicles or tubules) that incorporate TGBp2 and are delivered to the neck region of plasmodesmata and fused there to cortical ER (see Brandizzi et al., 2002; Cowan et al., 2002; Jiang et al., 2002; Solovyev et al., 2000; Stephens & Pepperkok, 2001; Vitale & Denecke, 1999). Therefore, TGBp3 may function as part of a membrane transport pathway for translocation of virus RNA, TGBp1 and TGBp2 to the orifices of plasmodesmata microchannels (see also Haywood et al., 2002; Lough et al., 1998; Lucas, 1999; Tamai & Meshi, 2001).

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REFERENCES

Detection of hordeivirus TGBp3

...potential role in synthesis of cellulose and callose in plants. Proc Natl Acad Sci U S A 92, 9353–9357.


