Subcellular localization of the Triple Gene Block movement proteins of *Beet necrotic yellow vein virus* by electron microscopy

M. Erhardt, G. Vetter, D. Gilmer, S. Bouzoubaa, K. Richards, G. Jonard, H. Guilley*

Institut de Biologie Moléculaire des Plantes du CNRS et de l’Université Louis Pasteur, 12 rue du Général Zimmer, 67084 Strasbourg, France

Received 18 May 2005; returned to author for revision 1 June 2005; accepted 7 June 2005
Available online 14 July 2005

Abstract

The Triple Gene Block proteins TGBp1, TGBp2, and TGBp3 of *Beet necrotic yellow vein virus* (BNYVV) are required for efficient cell-to-cell spread of the infection. The TGB proteins can drive cell-to-cell movement of BNYVV in trans when expressed from a co-inoculated BNYVV RNA 3-based ‘replicon’. TGBp2 and TGBp3 expressed from the replicon were nonfunctional in this assay if they were fused to the green fluorescent protein (GFP), but addition of a hemagglutinin (HA) tag to their C-termini did not incapacitate movement. Immunogold labeling of ultrathin sections treated with HA-specific antibodies localized TGBp2-HA and TGBp3-HA to what are probably structurally modified plasmodesmata (Pd) in infected cells. A similar subcellular localization was observed for TGBp1. Large gold-decorated membrane-rich bodies containing what appear to be short fragments of endoplasmic reticulum were observed near the cell periphery. The modified gold-decorated Pd and the membrane-rich bodies were not observed when the TGB proteins were produced individually in infections using the *Tobacco mosaic virus* P30 protein to drive cell-to-cell movement, indicating that these modifications are specific for TGB-mediated movement.

© 2005 Elsevier Inc. All rights reserved.

Keywords: Triple Gene Block; TGB; Cell to cell movement; Benyvirus; Tobacco mosaic virus

The spread of a plant virus infection from cell to cell in a host plant occurs via the plasmodesmata (Pd) and requires the intervention of one or more virus-coded movement proteins (MPs). For many viruses, the mobile entity for cell-to-cell movement is a ribonucleoprotein complex consisting of viral RNA, MP, and probably other proteins. *Tobacco mosaic virus* is the best studied example of a virus which employs such a cell-to-cell movement strategy (see Heinlein and Epel, 2004, for a review). Briefly put, the evidence indicates that the TMV 30K MP (P30) directs transport of the viral RNA–MP complex via the cytoskeleton to the vicinity of the Pd. Concurrently, P30 can augment the Pd size exclusion limit (SEL) and is believed to somehow participate in trafficking of the viral RNA through the altered Pd.

Sequence comparisons (Melcher, 2000) have revealed that many plant RNA viruses employ an MP of the P30 family, but other types of MP exist as well. For example, there is a large group of RNA viruses with rod-shaped or filamentous virions which possess a set of three MPs, none of which displays significant sequence similarity to the P30 family. These MPs are encoded by a cassette of three adjacent or slightly overlapping genes known collectively as the Triple Gene Block (TGB). The corresponding gene products are referred to (in 5' to 3' order along the genome) as TGBp1, TGBp2, and TGBp3 (see Morozov and Solovyev, 2003, for a recent review). TGBp1 is translated from a subgenomic RNA whose 5'-terminus lies upstream of the TGB1 cistron (Fig. 1). TGBp2 and TGBp3 are both translated from a shorter subgenomic RNA whose 5'-terminus lies upstream of the TGB2 gene (Fig. 1). The internal TGB3 gene on the shorter subgenomic RNA is probably accessed by leaky ribosome scanning (Skryabin et al., 1988; Morozov et al., 1989; Zhou and Jackson, 1996;...
The TGB-containing viruses can be divided into two classes. Class 1 consists of rod-shaped viruses of the genera *Hordeivirus*, *Pecluvirus*, *Pomovirus*, and *Benyivirus* and Class 2 of filamentous viruses of the genera *Potexvirus*, *Carlavirus*, *Foveavirus*, and *Allexivirus*. The Class 2 viruses require the viral coat protein (CP) in addition to either the CP or the TGBp1s have a molecular mass of ∼25 kDa and contain an NTP/helicase domain (Gorbalenya and Koonin, 1993). The TGBp1s of the Class 1 viruses are significantly longer than their counterparts in Class 2. They have an NTPase/helicase domain at their C-terminus but in addition possess an conserved hydrophobic domains of 19–23 amino acid residues (Morozov et al., 1989; Solovyev et al., 1996). By contrast, the TGBp3s of Class 1 and Class 2 viruses are dissimilar. The Class 2 TGBp3s have a molecular mass of 6–13 kDa and possess an N-terminal hydrophobic domain and a central hydrophilic region containing a conserved sequence motif with any of the other TGBp3s (Solovyev et al., 1996; Koenig et al., 1998), suggesting that it either has a different phylogenetic origin or has diverged dramatically since speciation.

The subcellular localization of autonomously expressed TGBp2 and TGBp3 of Class 1 viruses has been studied using GFP fusion constructs and confocal laser scanning microscopy (CLSM). Several different methods of delivering nucleic acid encoding the fusion protein into the plant cell have been employed including (i) biolistic delivery of DNA to epidermal cells (Solovyev et al., 2000; Zamyatin et al., 2002, 2004; Haupt et al., 2005), (ii) use of a heterologous virus as a vector (Cowan et al., 2002; Haupt et al., 2005), and (iii) production of transgenic plants (Gorshkova et al., 2003). In such experiments, the GFP-TGBp2 fusion protein was associated with the endoplasmic reticulum (ER) network and, in some cases, with ER-associated motile vesicles (Solovyev et al., 2000; Cowan et al., 2002; Zamyatin et al., 2004; Haupt et al., 2005). Motile vesicles containing GFP-TGBp2 of the pomovirus *Potato mop-top virus* (PMTV) which appeared at later times post-bombardment have been shown to contain plasma membrane and a marker for the endosomal pathway, leading to the suggestion that these bodies play a role in retrieving TGBp2 and TGBp3 from the cell membrane (Haupt et al., 2005).

Autonomous expression of GFP-TGBp3 led to the appearance of fluorescent membrane-proximal bodies of heterologous size at the cell periphery (Solovyev et al., 2000; Cowan et al., 2002; Zamyatin et al., 2002, 2004; Gorshkova et al., 2003; Haupt et al., 2005). The peripheral membrane-proximal bodies sometimes formed pairs opposite one another in adjacent cells (Cowan et al., 2002; Gorshkova et al., 2003) and colocalized with callose (Gorshkova et al., 2003), suggesting that they form...
preferentially at sites rich in intercellular connections, i.e., plasmodesmal pit fields.

When fluorescently labeled TGBp2 and TGBp3 were co-expressed in vivo, most of the TGBp2 was redirected from the ER network to peripheral membrane-proximal bodies resembling the structures formed upon autonomous expression of TGBp3 (Solovyev et al., 2000; Zamyatnin et al., 2002). At later times, TGBp2 of PMTV was also shown to relocalize TGBp3 to the aforementioned endocytic vesicles (Haupt et al., 2005). It is worth noting that BNYVV TGBp1 tagged with GFP also localized in a TGBp2- and TGBp3-dependent manner to putatively Pd-associated paired structures during a virus infection (Erhardt et al., 2000). Biolistic delivery of plasmids expressing PMTV GFP-TGBp1, TGBp2 and TGBp3 into epidermal cells resulted in the appearance of two types of fluorescent structures: large membrane-proximal bodies (“intermediate bodies”) and cell-wall-embedded punctate bodies (Zamyatnin et al., 2004). The GFP-TGBp1 was also observed to migrate into immediately adjacent cells. The intermediate bodies appear to be similar to the peripheral membrane-proximal bodies mentioned above which form following autonomous expression of GFP-TGBp3 or co-expression of GFP-TGBp2 and TGBp3. Co-expression of PMTV GFP-TGBp1 with various combinations of TGBp2 and TGBp3 from PMTV or other viruses revealed that the GFP-TGBp1 was localized to intermediate bodies by heterologous TGBp2 and TGBp3s but the cell-wall-embedded punctate bodies only formed when TGBp2 and TGBp3 were derived from PMTV (Zamyatnin et al., 2004).

Although the aforesaid studies with GFP-tagged proteins have provided useful insights into TGB-mediated cell-to-cell movement, one evident difficulty in their interpretation is that the GFP tag may interfere with the normal behavior of the protein of interest. While this does not appear to be the case with TGBp1, for which several studies have shown that GFP-TGBp1 retains its ability to drive cell-to-cell movement (Erhardt et al., 2000; Zamyatnin et al., 2004), it is a serious concern for the smaller TGB proteins since, to our knowledge, no TGBp2 or TGBp3 has been shown to retain its ability to mediate viral cell-to-cell movement when fused to GFP (although it should be noted that GFP-labeled TGBp2 and TGBp3 of PMTV are capable of increasing the local lesion formation on leaves of laboratory hosts such as Chenopodium quinoa but are required for virus infection in field conditions (see Jupin et al., 1991; Richards and Tamada, 1992). We have shown previously that one of these small RNAs, BNYVV RNA 3 (1.8 kb), can serve as an amplification-expression vector or ‘replicon’ for an inserted gene when the RNA 3 chimera is inoculated to leaves along with RNA 1 and 2 (Jupin et al., 1990).

In this paper, we have employed RNA 3-based replicons to determine if BNYVV TGBp2 and TGBp3 retain their function in cell-to-cell movement when fused either to GFP or to a 9 amino acid residue HA tag. In the case of TGBp2, the replicon transcripts to be tested were mixed with transcripts corresponding to RNA 1 and RNA 2I, an RNA 2 mutant with a frameshift mutation in the TGBp2 gene which disables the cell-to-cell movement function (see Fig. 1 for structures of the various RNA 2 mutants and replicons). The transcript mixtures were inoculated to C. quinoa leaves, and efficient cell-to-cell movement was judged to have occurred if local lesions were visible on the inoculated leaves by 7 days post-inoculation (pi) and progeny viral RNA was detected by Northern blot of a total RNA extract of the leaves. In all the following experiments, the appearance of local lesions was invariably accompanied by successful Northern blot detection of progeny viral RNA, and only the results of the Northern blot analysis will be shown.

As shown previously, inoculation of RNA 1 plus RNA 2I to C. quinoa leaves does not give rise to local lesions or amounts of progeny viral RNA detectable by Northern blot of RNA extracted from the inoculated leaves (Bleykasten-Grosshans et al., 1997; also see Fig. 2, lane 3). Addition of a replicon expressing wild-type TGBp2 (RepTGB2) to the inoculum, however, trans-complemented the defective cell-to-cell movement function, and progeny RNA 1, 2I, and RepTGB2 are readily detected by Northern blot (Bleykasten-Grosshans et al., 1997; Fig. 2, lane 4). Replicons expressing either N-terminal or C-terminal fusions of TGBp2 and GFP (RepGFP-TGB2 and
RepTGB2-GFP), on the other hand, failed the trans-complementation test, that is, no local lesions appeared on leaves inoculated with RNA 1, RNA 2I, and either replicon (data not shown), and no progeny viral RNA was detected by Northern blot (Fig. 2, lanes 5 and 6). Addition of an HA tag to the N-terminus of TGBp2 (RepHA-TGB2) likewise rendered the fusion protein inactive in cell-to-cell movement (Fig. 2, lane 7). By contrast, addition of the HA tag to the C-terminus of TGBp2 (RepTGB2-HA) did not interfere with the ability of the resulting fusion protein to trans-complement the movement function (Fig. 2, lane 8).

We have reported earlier that BNYVV TGBp3 can successfully trans-complement cell-to-cell movement when it is expressed from a dicistronic replicon (RepTGB2.3; Fig. 1) containing the TGB2 and TGB3 genes in the same relative configuration as in RNA 2 (Bleykasten-Grosshans et al., 1997). Consequently, the open reading frames of the TGBp3 fusion proteins to be tested (Fig. 1) were inserted into the RepTGB2.3 context. The replicons were mixed with transcripts of RNA 1 and RNA 2∆XE (Fig. 1), a deletion mutant targeting the TGB2 and TGB3 cistrons of RNA 2, and inoculated to C. quinoa leaves as before. As reported previously (Bleykasten-Grosshans et al., 1997), RepTGB2.3 trans-complemented the cell-to-cell movement defect of RNA 2∆XE (Fig. 2, lane 10). Successful trans-complementation was also observed for the dicistronic replicon RepTGB2.3-HA expressing HA-tagged TGBp3 (Fig. 2, lane 12), but not for the replicon RepTGB2.3-GFP which expresses TGBp3-GFP (Fig. 2, lane 11). We conclude that, as observed for most other TGB-containing viruses, fusion of a GFP tag to both BNYVV TGBp2 and TGBp3 abolishes their capacity to function in cell-to-cell movement. By contrast, addition of the smaller HA epitope tag to the C-terminus of either TGBp2 or TGBp3 does not compromise this function.

Cell-to-cell movement driven by a BNYVV RNA 2 chimera encoding the TMV P30 MP is not inhibited by autonomous expression of TGBp3 and TGBp3-HA

Earlier experiments revealed that a ‘monocistronic’ replicon expressing TGBp3 not only failed to trans-complement TGBp3-defective RNA 2 for cell-to-cell movement but also inhibited cell-to-cell movement when co-inoculated with wild-type BNYVV RNA (Bleykasten-Grosshans et al., 1997). As noted above, however, no such inhibitory effect was observed when TGBp3 was expressed from the dicistronic construct RepTGB2.3, which reproduces the relative positions of the TGB2 and TGB3 cistrons on the subgenomic RNA (Bleykasten-Grosshans et al., 1997; see also Fig. 2, lane 10). The reason why expression of TGBp3 from a monocistronic replicon inhibited cell-to-cell movement in these experiments is not known, but one possibility is that translation of TGBp2 and TGBp3 from the same messenger RNA provides a means of regulating the relative levels of accumulation of the two proteins, which could be critical for the movement function.

We have recently found that the BNYVV RNA 2 chimera RNA 2-2P30 (Fig. 1), in which all three TGB genes have been replaced by the P30 cistron, can drive BNYVV cell-to-cell movement. C. quinoa leaves inoculated with RNA 1 and RNA 2-2P30 transcripts developed local lesions of ~1 mm diameter by 7 days pi (data not shown), and progeny viral RNAs of the expected size were detected by Northern blot of RNA extracted from the leaves (Fig. 3, lane 2). When RepTGB1, RepTGB2, or RepTGB3 were included in the inoculum, they were replicated along with RNA 1 and RNA 2-2P30 (Fig. 3, lanes 3, 4, and 6), as were the replicons encoding the HA-tagged versions of TGB2 and TGB3 (Fig. 3, lanes 5 and 7). It is particularly noteworthy that co-inoculation of RepTGB3 (and RepTGB3-HA) with RNA 1 and RNA2-2P30 did not inhibit cell-to-cell movement, in contrast to the situation observed when the inoculum contained RNA 1, wild-type RNA 2, and RepTGB3 (Bleykasten-Grosshans et al., 1997). Thus, the use of RNA 2-2P30 to drive cell-to-cell movement provides a means of expressing TGBp3 and TGBp3-HA.
from a replicon without concurrent expression of TGBp1 and TGBp2.

Subcellular localization of BNYVV TGBp2-HA and TGBp3-HA

Although BNYVV TGBp2- and TGBp3-specific rabbit polyclonal antibodies are available (Niesbach-Klösgen et al., 1990), they have not been proven suitable for immunogold labeling studies because they produced unacceptable high background labeling on thin sections from healthy plant tissue (authors’ unpublished observations). This has led us to investigate the use of commercially available HA-specific mouse monoclonal antibodies to localize the HA-tagged versions of BNYVV TGBp2 and TGBp3 in thin sections. For detection of TGBp2-HA, *C. quinoa* leaves were inoculated with transcripts of BNYVV RNA 1, RNA 2I, and RepTGB2-HA. Tissue was taken from the leading edge of the resulting local lesions at 7 days pi and prepared for transmission electron microscopy. The fixed sections were treated with HA-specific mouse monoclonal antibody, and the immunolabeled sites were then decorated by treatment with goat anti-mouse (GAM) IgGs which had been conjugated to 15 nm colloidal gold beads and examined by transmission electron microscopy (TEM). For the experiments aimed at detecting TGBp3-HA, the inoculation mix contained transcripts of RNA 1, RNA 2ΔXE, and RepTGB2.3-HA. Sample preparation from *C. quinoa* leaves inoculated with the RepTGB2.3-HA-contain-

ing transcript mixture was carried out exactly as described for the inoculum containing RepTGB2-HA. As a control, tissue taken from healthy *C. quinoa* was also carried through the sample preparation protocol.

In a first series of observations, each set of thin sections was examined at low magnification, and at least 50 randomly selected cell-wall-associated structures, which we will refer to as ‘paramural bodies’ (Marchant and Robards, 1968), were scored for the presence of immunogold decoration. In the sections from the RepTGB2-HA-inoculated tissue, 24 of the observed paramural bodies were gold-decorated (48%), while in the sections from the RepTGB2.3-HA-inoculated tissue, 21 of 51 (41%) of the structures were gold-decorated (see Figs. 4A, panel 1 and B, panel 1 for typical images and Table 1 for a summary of the data). In the sections obtained from tissue infected with RNA 1 plus 2P30, only 2–3% of the paramural bodies observed (all of which were Pd) were associated with gold beads. These observations provide direct evidence that biologically functional TGBp2 and TGBp3 are targeted to cell-wall-associated structures during a BNYVV infection when TGBp1 is present.

Interestingly, most (60–70%) of the paramural bodies observed during the sweep of the RepTGB2-HA- and the RepTGB2.3-HA-inoculated tissue were not typical Pd but were regions in which the cell wall had undergone a pronounced thickening so that it bulged out into the cytoplasm. Osmiophilic deposits were generally present in the thickened region (see Figs. 4A, panel 1 and B, panel 1, for examples). These cell wall thickenings were only present in sections from infected tissue in which the genes for all three TGB proteins were included in the inoculum. They were never observed in healthy tissue (data not shown) or in tissue infected with a transcript mix in which one or more of the TGB genes were absent: in such tissue, only normal Pd were observed (e.g., Figs. 4A and B, panels 3 and 5).

To gain further information concerning the origin of the cell wall thickenings, we carried out immunogold-labeling experiments with a mouse monoclonal antibody specific for callose, a marker for Pd (Radford et al., 1998). The BNYVV infection-specific cell wall thickenings were heavily gold-decorated with this antibody (Fig. 5, panels 2–4). This is consistent with, but does not definitively prove, the hypothesis that the paramural bodies are Pd which have undergone extensive modification during the infection, presumably due to the presence of the TGB proteins.

In addition to the aforesaid cell wall thickenings, the thin sections from the tissue infected with RNA 1, RNA 2I, and RepTGB2-HA or with RNA 1, RNA 2ΔXE, and RepTGB2.3-HA often contained a very large irregularly shaped electron-dense region (Figs. 4A, panel 2, and B, panel 2) which we will refer to as a membrane-rich peripheral body (MRPB). Such structures were never observed in sections from healthy tissue (data not shown), and they stained differently from nuclei (see Fig. 5 panels 5 and 6). The interior of the MRPBs contained numerous...
Fig. 4. Subcellular localization of TGBp1, TGBp2-HA, and TGBp3-HA in cells in the leading edge of local lesions on infected C. quinoa leaves. (A) Thin sections were taken from leaves inoculated with RNA 1, RNA 2, and RepTGB2-HA (1 and 2), RNA 1 and RNA2 2P30 (3 and 4), or RNA 1, RNA 2P30, and RepTGB2-HA (3 and 4), or RNA 1 and RNA2 2P30 (5 and 6). All the sections were treated with 15 nm colloidal gold-labeled goat-anti-mouse antibodies. Colloidal gold particles are indicated by arrows in some cases. Scale bar corresponds to 500 nm. In the insert in panel 2, the membrane-rich peripheral body (MRPB) is shown at reduced magnification, and its contours are outlined. (B) The thin sections were taken from leaves inoculated with transcripts of RNA 1, RNA 2, and RepTGB2.3-HA (1 and 2), RNA 1, RNA 2P30, and RepTGB3-HA (3 and 4), or RNA 1 and RNA2 2P30 (5 and 6). The thin sections were immunolabeled with HA-specific antibodies as in panel A. Other details as in panel A. CW: cell wall; ER: endoplasmic reticulum; Pd: plasmodesma; MPD: modified Pd (i.e., cell wall structures characterized by cell wall thickening and osmiophilic bodies (Os); MRPB: membrane-rich peripheral body.
short segments of bilayer membrane which resemble fragmented and disorganized ER. Sometimes, the bodies appeared to be separated from the surrounding cytoplasm by a more or less continuous ring of ER (e.g., Fig. 5, panels 5 and 6), but this was not always the case (see Fig. 4A, panel 2; Fig. 4B, panel 2; and Fig. 4C panel 2). The MRPBs were generally situated near the cell wall, but no obvious physical connection with Pd was apparent. We have noted, however, that in several cases the ER network in the immediate vicinity of the cell wall thickenings mentioned above was also disorganized and fragmented (e.g., Fig. 4A, panel 1) in a similar manner. The MRPBs were immunogold-labeled with the TGBp2-HA- and TGBp3-HA-specific antisera (Figs. 4A, panel 2, B, panel 2). The number of gold beads per μm² in the MRPBs was 8–10 times higher in the TGBp2-HA- and TGBp3-HA-containing thin sections than

Table 1

<table>
<thead>
<tr>
<th>Antigen detected</th>
<th>Transcripts inoculated</th>
<th>TGB proteins synthesized</th>
<th>% labeled paramural bodies</th>
</tr>
</thead>
<tbody>
<tr>
<td>TGBp1</td>
<td>RNA1 + 2</td>
<td>TGBp1, TGBp2, TGBp3</td>
<td>48</td>
</tr>
<tr>
<td></td>
<td>RNA1 + 2P30 + RepTGB1</td>
<td>TGBp1</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>RNA1 + 2P30</td>
<td>none</td>
<td>2</td>
</tr>
<tr>
<td>TGBp2-HA</td>
<td>RNA1 + 2 + RepTGB2-HA</td>
<td>TGBp1, TGBp2-HA, TGBp3</td>
<td>48</td>
</tr>
<tr>
<td></td>
<td>RNA1 + 2P30 + RepTGB2-HA</td>
<td>TGBp2-HA</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>RNA1 + 2P30</td>
<td>none</td>
<td>3</td>
</tr>
<tr>
<td>TGBp3-HA</td>
<td>RNA1 + Δ2XE + RepTGB2.3-HA</td>
<td>TGBp1, TGBp2, TGBp3-HA</td>
<td>41</td>
</tr>
<tr>
<td></td>
<td>RNA1 + 2P30 + RepTGB3-HA</td>
<td>TGBp3-HA</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>RNA1 + 2P30</td>
<td>none</td>
<td>3</td>
</tr>
</tbody>
</table>

At least 50 paramural bodies were inspected for each type of infection, and the percentage of labeled structures is indicated.
in the surrounding cytoplasm (Table 2), indicating that TGBp2 and TGBp3 accumulate preferentially at these sites.

_TGBp2-HA and TGBp3-HA are not targeted to the Pd when expressed without the other TGB proteins_

In the foregoing experiments, the genes encoding all three BNYVV TGB proteins were present in the inoculum. To determine whether TGBp2 and TGBp3 are targeted to the Pd when they are expressed individually, RepTGB2-HA or RepTGB3-HA were inoculated to _C. quinoa_ leaves with RNA 1 and RNA 2P30 transcripts. Tissue from the leading edge of the resulting local lesions was thin-sectioned and visualized by TEM as described above. For both types of inoculum, immunogold labeling was observed in the cytoplasm, sometimes in association with the ER (Fig. 4A, panels 3 and 4; Fig. 4B, panels 3 and 4), but no significant labeling of the Pd occurred (see Table 1).
Results are the average from observations on three thin sections.

Furthermore, the cell wall thickenings that were apparent when all three TGB proteins were expressed were never observed, although Pd were abundant (Figs. 4A panel 3 and B, panel 3). These Pd resembled the Pd characteristic of healthy tissue (data not shown) and of tissue infected with RNA 1 plus RNA 2P30 (Figs. 4A, panel 5 and B, panel 5). No MRPBs were observed in any of these thin sections, indicating that formation of these structures likely requires the entire complement of TGB proteins.

BNYVV TGBp1 also localizes to Pd and MRPBs

We have previously used immunogold labeling techniques to show that the TGBp1 of the Class 1 virus Peanut clump virus (PCV, genus Pecluvirus) localizes to Pd during a virus infection (Erhardt et al., 1999). We have now carried out a similar study for the TGBp1 of BNYVV. C. quinoa was inoculated with BNYVV RNA 1 and RNA 2 transcripts. Tissue samples were taken from the leading edge of local lesions at 7 days pi, and ultrathin sections were prepared as described above. The sections were treated with a rabbit polyclonal antibody specific for BNYVV TGBp1 (Niesbach-Klösgen et al., 1990), and sites of antibody fixation were labeled by treatment with goat anti-rabbit (GAR) IgGs conjugated to 15 nm colloidal gold beads. TEM observations such as those described above revealed that TGBp1, like TGBp2-HA and TGBp3-HA, also localized to structurally modified Pd and to MRPB (see Fig. 4C, panels 1 and 2; see also Tables 1 and 2). When TGBp1 was expressed from a replicon (RepTGBp1) in an inoculum containing transcripts of RNA 1 and RNA 2P30, immunogold labeling was distributed in a uniform manner throughout the cytoplasm, and no specific labeling of Pd was observed (Fig. 4C, panels 3 and 4, and Table 1), in line with our previous findings using GFP-tagged protein which showed that BNYVV GFP-TGBp1 requires both TGBp2 and TGBp3 to localize to the Pd (Erhardt et al., 2000).

Discussion

The RNA 3 replicon system has been previously used to show that the BNYVV TGB proteins can function outside of their normal context in RNA 2. Thus, RNA 3-based replicons encoding TGBp1, TGBp2, or both TGBp2 and TGBp3 (expressed from a dicistronic construct) can provide movement functions in trans when co-inoculated to C. quinoa with transcripts of RNA 1 plus an RNA 2 mutant in which the copy of the corresponding TGB gene or genes had been disabled (Bleykasten-Großhans et al., 1997). Likewise, an RNA 3-based replicon expressing a BNYVV GFP-TGBp1 fusion protein could trans-complement the BNYVV cell-to-cell movement function when the TGB1 gene on RNA 2 had been disabled by mutation (Erhardt et al., 2000). Confocal laser scanning microscopy (CLSM) of local lesions on leaves infected with such a transcript mixture allowed us to localize the GFP-TGBp1 to cell-wall-embedded punctate bodies (Erhardt et al., 2000).

In this paper, we have employed the RNA 3-based replicon system to express HA-tagged rather than GFP-tagged versions of BNYVV TGBp2 and TGBp3 during a BNYVV infection. Use of the replicon as a gene expression vehicle rather than inserting the tag sequence into RNA 2 could interrupt cis-acting replication signals or other important sequences. With the replicon system, we have shown that, in contrast to GFP-tagged TGBp2 and TGBp3, TGBp2-HA and TGBp3-HA retain their ability to drive BNYVV cell-to-cell movement in C. quinoa, permitting study of their subcellular localization under conditions approximating an infection with wild-type BNYVV.

Current models of Class 1 TGB-mediated cell-to-cell movement have suggested that TGBp2 and TGBp3 act to traffic and/or dock TGBp1-RNA ‘movement’ complexes to the Pd (Morozov and Solovyev, 2003; Haupt et al., 2005). Here, we show that BNYVV TGBp2-HA and TGBp3-HA both localize to callose-containing cell wall thickenings which we believe represent degenerate Pd. Interestingly, cell wall thickenings associated with osmiophilic deposits similar to those reported here have also been observed in BSMV-infected barley (McMullen et al., 1977), illustrating that the appearance of such structures is not limited to BNYVV. The cell wall thickenings in the BSMV-infected barley were closely associated with structures which traversed the cell wall and which appeared to be desmotubules (McMullen et al., 1977). The analogous structures observed by us did not contain desmotubules traversing the cell wall, although they often contained short, irregularly oriented bilayer structures which could be desmotubular fragments (e.g., Fig. 4A, panel 1). In view of their apparent lack of a desmotubule, it is unlikely that the highly modified Pd viewed in the BNYVV-infected tissue can support intercellular movement of virus or solutes. Whether the degeneration of the desmotubules and the other modifications is a direct consequence of the presence of the TGB proteins in the structures or an indirect response (e.g., the result of a
plant defense reaction aimed at slowing down viral cell-to-cell movement) is an open question. Our finding that BNYVV TGBp2-HA and TGBp3-HA can be detected at structures which are probably highly modified Pd is consistent with the numerous lines of evidence that these two proteins are at least transiently associated with Pd. Haupt et al. (2005) recently provided evidence that these two proteins are at least transiently modified Pd is consistent with the numerous lines of evidence that these two proteins are at least transiently modified, which may deliver them back to the perinuclear membrane where they could re-enter the virus transport pathway. We did not detect such endocytic vesicles in our electron micrographs, suggesting that such a recycling pathway, if it occurs in BNYVV-infected cells, does not involve large numbers of vesicles and/or that such vesicles are produced only at times during the infection process which were not sampled in our analyses. The fact that neither TGBp2-HA nor TGBp3-HA are targeted to the Pd when expressed autonomously (in infections in which cell-to-cell movement was mediated by P30) suggests that targeting of these proteins to the Pd, and/or their accumulation there, requires the full complement of BNYVV TGB proteins, in line with our earlier observations (Erhardt et al., 2000). Likewise, the Pd-associated cell wall thickenings provoked by BNYVV infection did not occur when cell-to-cell movement was mediated by P30, even if TGBp1, TGBp2, or TGBp3 was concurrently expressed from a co-inoculated replicon. Thus, the modifications are apparently specific for TGB-mediated cell-to-cell movement and are not provoked by BNYVV infection per se. We cannot, however, strictly rule out the possibility that co-expression of P30 with the different TGB proteins in our experiments somehow prevents the latter from making their way to the Pd and provoking structural modifications there. The newly discovered MRPs described here were not observed in our previous electron microscopic observations of BNYVV-infected cells (Erhardt et al., 2001), presumably because the tissue fixation and staining protocols used in the earlier study were designed to optimize immunogold labeling rather than visualization of membranous structures. It should be noted that formation of the MRPB requires expression of all three TGB proteins, as such structures were not detected in BNYVV infections when P30 was used to drive cell-to-cell movement. This finding argues against the possibility that the MRPs are essential structures for BNYVV RNA replication. Immunogold labeling showed that BNYVV TGBp1, TGBp2-HA, and TGBp3-HA were present in the MRPs. Other workers have shown that autonomously expressed GFP-TGBp3 of the Class 1 viruses *Poa semilatent virus* (PSLV) and PMTV accumulate in ER-rich membrane bodies near the cell periphery, and both PSLV and PMTV GFP-TGBp2 were redirected to similar structures when co-expressed with a homologous or heterologous TGBp3 (Solovyev et al., 2000; Zamyatnin et al., 2002, 2004; Gorshkova et al., 2003). These structures may be related to the MRPs observed in this study. It should be noted, however, that, in contrast to the aforesaid experiments, we did not detect MRPs following expression of TGBp3-HA from a replicon co-inoculated with RNA 1 plus RNA 2P30, i.e., under conditions in which TGBp3 is expressed independently of other TGB proteins. Possibly, the failure to produce MRPs under these circumstances is due to differences in the level or kinetics of expression of TGBp3 from the replicon compared to the situation when GFP-TGBp3 is transiently expressed from a biolistically delivered DNA. Further experimentation will be required to establish the relationship between the MRPs described here and the peripheral membrane bodies described by other workers. It will also be of interest to determine what role, if any, the MRPs play in cell-to-cell movement of BNYVV.

**Materials and methods**

**Clones and infectious transcripts**

cDNA clones of BNYVV RNA 1, RNA 2–14, RNA 2I, and RNA 2ΔXE and the replicons Rep0, RepTGB1, RepTGB2, RepTGB3, and RepTGB2.3 have been described elsewhere (Bleykasten-Grosshans et al., 1997). Fusions between TGBp2 or TGBp3 and GFP (Reichel et al., 1996) or the hemagglutinin (HA) tag sequence YPPDVPDYA (Wilson et al., 1984) were generated by overlap extension mutagenesis (Ho et al., 1989) of cloned cDNA during PCR. In RepGFP-TGB2, the GFP termination codon was replaced by CCC and the TGB2 initiation codon by GGG. In RepTGB2-GFP, the TGB2 termination codon was replaced by CCC and the GFP initiation codon by GGG. The same approach was used to create the dicistronic construct RepTGB2.3-GFP.

The cDNA encoding the TGB2p2 protein was also fused with either an N-terminal or C-terminal HA tag by PCR using appropriate TGB2-specific primers to which the HA DNA sequence had been appended. The primers for fusion of the tag to the N-terminus of TGBp2 were 5'-ATTAGGATCCATGTACCCATAGCAGCTCAGCT-3' and 5'-ATTAGGATCCATGTACCCATAGCAGCTCAGCT-3'. The primers for fusion of the tag to the C-terminus were 5'-ATTAGGATCCATGTACCCATAGCAGCTCAGCT-3' and 5'-ATTAGGATCCATGTACCCATAGCAGCTCAGCT-3' (BamHI sites are in italics, and the sequences encoding the HA tag are underlined). A similar approach was used to create PCR fragments in which the HA tag was fused to the C-terminus of TGBp3. The resulting PCR fragments were cleaved with *BamHI* and inserted into Rep0 which had been linearized with *BamHI* and treated with alkaline phosphatase. Clones containing the PCR fragment in the appropriate orientation were identified by restriction.
enzyme digestion, and the insert was certified to be error-
free by sequence analysis.

To create a BNYVV RNA 2 construct carrying the TMV P30 gene, a PCR fragment was produced containing the TMV common strain P30 gene (template cDNA provided by Anne Berna) flanked by a 5’-terminal NcoI site and a 3’-terminal EcoRI site. The TGB1 initiation codon sequence in the RNA 2 transcription vector pb2-14 was converted into an NcoI site by overlap extension mutagenesis to produce pb2-14N. The NcoI–EcoRI-cleaved P30 PCR fragment was inserted into pb2-14N between this novel NcoI site and an EcoRI site in the TGB3 gene to create RNA 2P30 (see Fig. 1).

Preparation of transcripts and inoculation

Capped transcripts were prepared with a Ribomax transcription kit (Promega), and recombinant plasmids linearized with HindIII (for plasmids producing RNA 1 and the replicons) or SalI (RNA 2 and its derivatives). The amount of full-length transcript was evaluated by agarose gel electrophoresis, and the transcripts (5 μg RNA 1 and RNA 2, 2 μg replicon) were diluted in 100 μl 50 mM potassium phosphate, pH 7.5, and mechanically inoculated to celite-dusted C. quinoa leaves (Quillet et al., 1989).

Detection of viral RNA

Total RNA was isolated from plants 5–8 days pi and subjected to Northern hybridization after denaturing agarose gel electrophoresis and transfer to nitrocellulose (Gilmer et al., 1992). RNA 1 and RNA 2 were detected with previously described 32P-labeled antisense RNA probes (Bleykasten-Grosshans et al., 1997), and the different replicons were detected with a 32P-labeled antisense RNA probe complementary to nucleotides 1–380 of RNA 3.

Immunolocalization of the TGB proteins by transmission electron microscopy

Leaf tissue samples were taken from the leading edge of local lesions at 5–7 days pi and were fixed overnight in 4% glutaraldehyde and were then treated for 2 h with 10% (w/v) picric acid, 2 h with 2% uranyl acetate and then stained with 0.1% (v/v) osmium tetroxide in 150 mM phosphate buffer, pH 7.2. Samples were dehydrated through an ethanol series and infiltrated with EPON812 medium grade resin (Poly-science, Germany). Polymerization was for 48 h at 60 °C. Ultrathin sections (90 μm) were cut using an ultracut E microtome (Reichert) and collected on grids coated with formvar (EMS, Washington). For localization of BNYVV TGBP1, the sections were incubated with a previously described TGBP1-specific rabbit polyclonal antibodies (Niesbach-Klösgen et al., 1990) for 2 h at room temperature. After repeated washing with PBS, the sections were then incubated for 2 h with goat-anti-rabbit antibodies coupled to 15 nm colloidal gold particles (Aurion EM Reagents, the Netherlands) followed by washing with PBS and H2O. Samples were visualized with a Hitachi H-600 electron microscope operating at 75 kV. Samples for immunodetection of BNYVV TGBp2-HA and TGBp3-HA were prepared for electron microscopy as described above except that the primary immunolabeling reaction was with HA-specific mouse monoclonal antibodies (Aurion EM Reagents, The Netherlands), and the secondary reaction used goat anti-mouse antibodies conjugated to 15 nm colloidal gold particles (Aurion EM Reagents, the Netherlands). Immunodetection of callose was performed in a similar manner except the primary reaction employed mouse monoclonal antibodies specific for (1–3)β-D-glucan (Biosupplies, Australia).

Acknowledgments

We thank Christophe Ritzenthaler for the callose-specific antibodies and advice. The Inter-institute Microscopy Platform used in this study was cofinanced by the Région Alsace, the CNRS, the Université Louis Pasteur, and ARC.

References


