Barley stripe mosaic virus-encoded proteins triple-gene block 2 and γb localize to chloroplasts in virus-infected monocot and dicot plants, revealing hitherto-unknown roles in virus replication

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Replication of *Barley stripe mosaic virus* (BSMV), genus *Hordeivirus*, is thought to be associated with vesicles in proplastids and chloroplasts, but the molecular details of the process and identity of virus proteins involved in establishing the virus replication complexes are unknown. In addition, BSMV encodes a triple-gene block of movement proteins (TGBs) that putatively share functional roles with their counterparts in other hordei-, pomo- and pecluviruses, but detailed information on the intracellular locations of the individual TGBs is lacking. Here, the subcellular localizations of BSMV-encoded proteins TGB2 and γb fused to green or red fluorescent proteins were examined in epidermal cells of *Nicotiana benthamiana* and barley (*Hordeum vulgare* 'Black Hulless'). The fusion proteins were expressed from a BSMV vector or under the control of the cauliflower mosaic virus 35S promoter. The subcellular localizations were studied by confocal laser-scanning microscopy (CLSM). CLSM studies showed that both proteins were recruited to chloroplasts in the presence of viral RNA and that virus RNA, coat protein and γb protein were detected in plastid preparations from infected leaves. Electron microscope images of thin sections of virus-infected leaves revealed abnormal chloroplasts with cytoplasmic inclusions containing virus-like particles. In addition, cellular localizations of BSMV TGB2 suggest subtle differences in function between the hordei-like TGB2 proteins. The results indicate that TGB2 and γb proteins play a previously unknown functional role at the site of virus replication.

INTRODUCTION

Positive-sense single-stranded RNA (ssRNA) virus replication occurs in association with intracellular membranes of different origins, including the endoplasmic reticulum (ER) (e.g. *Brome mosaic virus*; BMV), multivesicular bodies derived from mitochondria, peroxisomes or vacuoles (e.g. *Cucumber mosaic virus*) and chloroplasts (e.g. *Turnip yellow mosaic virus*) (reviewed by Ahlquist *et al.*, 2003; Salonen *et al.*, 2005). Viral proteins associated with replication co-localize with viral genomic RNA and host factors to form a replication complex that is assembled on intracellular membranes. Often the membranes are invaginated to create compartments that bring together the components of replication (Ahlquist *et al.*, 2003); such compartmentalization helps to isolate them from host-cytoplasmic components and, arguably, may also help to avoid RNA-mediated defence responses (Ahlquist *et al.*, 2003). Replication of *Barley stripe mosaic virus* (BSMV) is associated with plastids (Carroll, 1970; Lin & Langenberg, 1985), but the identity of the viral proteins involved and how the complex is recruited to the plastid are unknown.

The BSMV genome contains a triple-gene block of movement proteins (TGBs) (Fig. 1). TGB-containing viruses have been classified into two groups: the hordei-like group, including BSMV and *Potato mop-top virus* (PMTV), and the

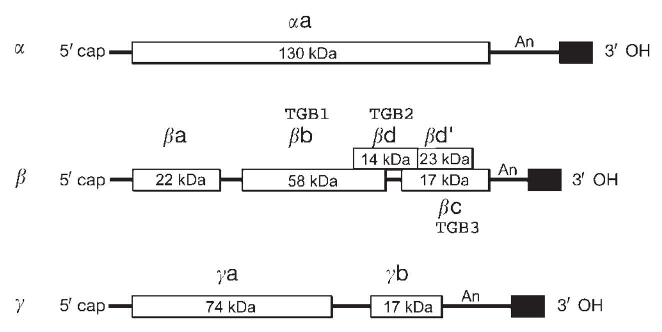


Fig. 1. Diagram of BSMV genome organization; the mass of the encoded proteins is given within the boxes. Triple gene-block proteins 1, 2 and 3 are indicated on RNA β. Solid rectangles indicate tRNA-like structures.

potex-like group, including *Potato virus X* (PVX) (reviewed by Morozov & Solovyev, 2003). Although the genetic arrangement of overlapping reading frames of the TGBs in the two groups is well conserved, there is some variation in both molecular mass and genome position, particularly within the hordei-like TGBs. The TGB1 protein of hordei-like viruses contains conserved helicase motifs found in the TGB1 protein of potex-like viruses and a large N-terminal domain thought to be involved in RNA binding. Each of the TGBs is required for virus movement, but, unlike the potex-like viruses, the hordei-like viruses do not require coat protein (CP) for cell-to-cell movement (Morozov & Solovyev, 2003).

For viruses with hordei-like TGBs, it is thought that TGB1 interacts with RNA and forms part of the ribonucleoprotein (RNP) complex; green fluorescent protein (GFP)-tagged TGB1 requires the presence of TGBs 2 and 3 for localization to the plasma membrane and plasmodesmata (PD) (Lawrence & Jackson, 2001a; Zamyatnin *et al.*, 2004). TGBs 2 and 3 are integral membrane proteins that act together to deliver the RNP complex from the site of virus replication to the PD by a vesicle-mediated transport process (Haupt *et al.*, 2005). Experiments using fluorescent protein-tagged PMTV TGBs have revealed that TGBs 2 and 3 co-localize in cellular membranes and mobile granules, utilize the actin–ER network to facilitate movement to the cell periphery and PD and associate with components of the endocytic pathway. There was no evidence of association with microtubules and the results suggest that TGBs 2 and 3 do not move from cell to cell (Haupt *et al.*, 2005).

Many virus-encoded proteins are multifunctional and it has been shown that PVX TGB1 suppresses the host-defence RNA-silencing response (Voinnet *et al.*, 2000), whereas in BSMV, the cysteine-rich, 17 kDa γb protein displays RNA-binding and silencing-suppressive activities (Donald & Jackson, 1996; Bragg *et al.*, 2004). These findings suggest that the BSMV γb protein plays a role in virulence and counter-defence during BSMV infection in monocot and dicot hosts (Bragg *et al.*, 2004).

We have used transient-expression vectors and BSMV reporter clones that express fluorescent proteins to study the movement and localizations of BSMV γb and TGB2 proteins and to investigate whether intracellular associations similar to those found with PMTV could be observed. The results reveal that there are differences in localizations of BSMV and PMTV GFP-tagged TGB2s. In addition, BSMV γb and TGB2 localize to chloroplasts that also contain viral RNA and virus-like particles, revealing previously unknown roles for these proteins in supporting virus replication.

METHODS

Reporter clones and plasmids. BSMV cDNA clones were based on the parent ND18 strain of BSMV (Petty *et al.*, 1988). The plasmid encoding RNA γ was modified so that GFP was fused to the C terminus of γb (RNA γ–γb–GFP; Lawrence & Jackson, 2001b). These cDNA clones were linearized and used as templates for production of infectious transcripts under the control of a T7 promoter. In addition, BSMV clones were constructed under the control of the cauliflower mosaic virus 35S promoter (P_{35S}). Here, the RNA γ was modified so that the gene encoding γb was fused in frame to the sequences of the foot-and-mouth virus 2A peptide (Ryan *et al.*, 1991) and GFP, so that, on expression *in vivo*, a proportion of the γb protein remained fused to GFP at the N terminus (RNA γ–GFP–2A–γb; G. Pogue, personal communication).

The gene encoding BSMV TGB2 was fused to the 3′ terminus of the GFP sequence and then cloned into the plasmid vector pRTL2 under the control of P_{35S} (35S–GFP–TGB2).

The 35S-monomeric red fluorescent protein (mRFP)–γb and 35S–GFP–γb plasmids were prepared by using the Gateway system (Invitrogen). The BSMV γb gene was amplified by PCR and cloned into the Gateway entry vector pDONR221 before recombination with Gateway-compatible pRTL2–mRFP or pRTL2–GFP (prepared according to the manufacturer's instructions).

Confocal laser-scanning microscopy (CLSM). Plasmid DNA or virus transcripts were introduced into epidermal cells of *Nicotiana benthamiana* or barley (*Hordeum vulgare* 'Black Hulless') by biolistic bombardment. The cells were examined by CLSM after 1–2 days as described by Haupt *et al.* (2005).

Electron microscopy (EM). Sections taken from BSMV-infected leaves were fixed in 5 % (w/v) glutaraldehyde in PIPES buffer containing 1 % tannic acid. After fixation, some of the sections were also post-fixed in 0·2 % (w/v) osmium tetroxide before dehydration and embedding in LR White resin (Oparka *et al.*, 1999). Ultrathin sections were mounted on pyroxylin-coated nickel grids, post-stained with uranyl acetate and lead citrate and examined by using a Phillips CM10 electron microscope.

Plastid preparations. Plastids were isolated from infected barley leaves essentially as described by Robinson (1994). Briefly, leaves were triturated in 5 vols sorbitol medium [50 mM HEPES/KOH (pH 8·4), 0·33 M sorbitol] and filtered through muslin. The extract was centrifuged at 4000 ***g*** for 1 min and the resulting pellet was resuspended in sorbitol medium (0·2 vol. starting material) and then layered onto an equal volume of 40 % Percoll (prepared in sorbitol medium). Following centrifugation at 2500 ***g*** for 7 min (with the brake off), the pellet containing the plastids was resuspended in sorbitol medium.

RT-PCR. Total RNA extraction, DNase I treatment and reverse transcription from leaf samples or chloroplasts prepared from mock- and BSMV-infected barley leaves were done as described previously (Lacomme *et al.*, 2003). Synthesis of the first strand from the genomic and subgenomic positive-strand BSMV RNA γ was achieved by using an antisense primer (5′-TAAATTTTCTCTCC-AGAGTCCGTTAAGATTC-3′) and 1 μg DNase I-treated total RNA. Forward and reverse primers, respectively 5′-GTTAACGCAATA-CGGTAAG-3′ and 5′-AGTTCGATTATAGTGGAC-3′, were used to amplify a 200 nt fragment of the γ cDNA.

Western blots. Samples of leaf extracts and plastid preparations were electrophoresed by SDS-PAGE (12·5 % gel), the proteins were electroblotted onto Hybond ECL nitrocellulose membrane and the membrane was subsequently incubated with antibody preparations essentially as described previously (Torrance, 1992). Rabbit antiserum to BSMV CP was used at a dilution of 1/1000 and mouse antiserum to γb at 1/500, followed by anti-mouse or anti-rabbit–alkaline phosphatase conjugate (Sigma A8025 or A1902).

RESULTS

GFP-tagged γb protein, when expressed from reporter virus clones, remained cytosolic, but also localized to chloroplasts

BSMV reporter clones for the expression of GFP-tagged γb (a preparation of RNA α, RNA β and either RNA γ–GFP–2A–γb or RNA γ–γb–GFP) were delivered by particle bombardment into leaves of *N. benthamiana* or barley and the epidermal cells were examined by CLSM. In these experiments, the intracellular localization of fluorescence was identical, irrespective of whether the clones expressed GFP–2A–γb or γb–GFP. In *N. benthamiana* epidermal cells, GFP–2A–γb was mainly seen in the cytoplasm and was also localized to discrete, round, vesicle-like compartments approximately 4 μm in diameter (arrow in Fig. 2a; Fig. 2b). The vesicle-like compartments were often seen clustered around the nucleus (Fig. 2a), with GFP–2A–γb forming a ring surrounding small, discrete patches of fluorescence within (Fig. 2b). For detection of GFP, the epidermal cells were imaged by using 488 nm excitation with emission collected between 500 and 530 nm. However, a lambda scan of the vesicle-like compartments, from 595 to 755 nm at 5 nm intervals, revealed a second emission peak between 650 and 700 nm, distinct from the GFP emission peak. The signal for this second peak was found to be from within the vesicle-like compartments (Fig. 2c). The spectral characteristics of this emission peak matched the emission peak of chlorophyll *a* autofluorescence exactly (maximum at 680 nm; Fig. 2e). The fluorescence emission from the vesicles was collected simultaneously, with one photon multiplier-tube bandwidth set at 500–530 nm and a second one at 660–700 nm. This revealed that the GFP–2A–γb-labelled vesicle-like compartments contained chlorophyll, although the chlorophyll did not fill the vesicles completely (Fig. 2b–d). We believe that these compartments are plastids, probably chloroplasts, and that GFP–2A–γb is associated with the chloroplast envelope and also localized to discrete patches within the chloroplasts. GFP–2A–γb was also observed in the membranes of similarly sized round vesicles that did not contain chlorophyll; these may be another population of plastids, for example leukoplasts, or another vesicle population.

Transient expression of the reporter clones in barley epidermal cells also revealed that GFP–2A–γb was localized in the cytoplasm and was found to be associated with ring-like structures that moved along cytoplasmic strands. These structures were of variable diameter (4–8 μm), but were not always round; they had a more pleiomorphic shape. They moved within the flow of the cytoplasm and did not contain chlorophyll (arrow in Fig. 2f). Absence of labelled chloroplasts was not surprising, as barley epidermal cells generally do not contain chloroplasts; we believe that, in this cell type, GFP–2A–γb is associated with the membrane of a subpopulation of vesicles within the cytoplasm. However, in barley mesophyll cells, GFP–2A–γb was localized both in the cytoplasm and, as described in *N. benthamiana* cells, to plastids that were approximately 4 μm in diameter and contained both chlorophyll and internal spots of GFP–2A–γb (arrow in Fig. 2g).

Virus-expressed GFP–2A–γb localized to chloroplasts independently of TGB and CP

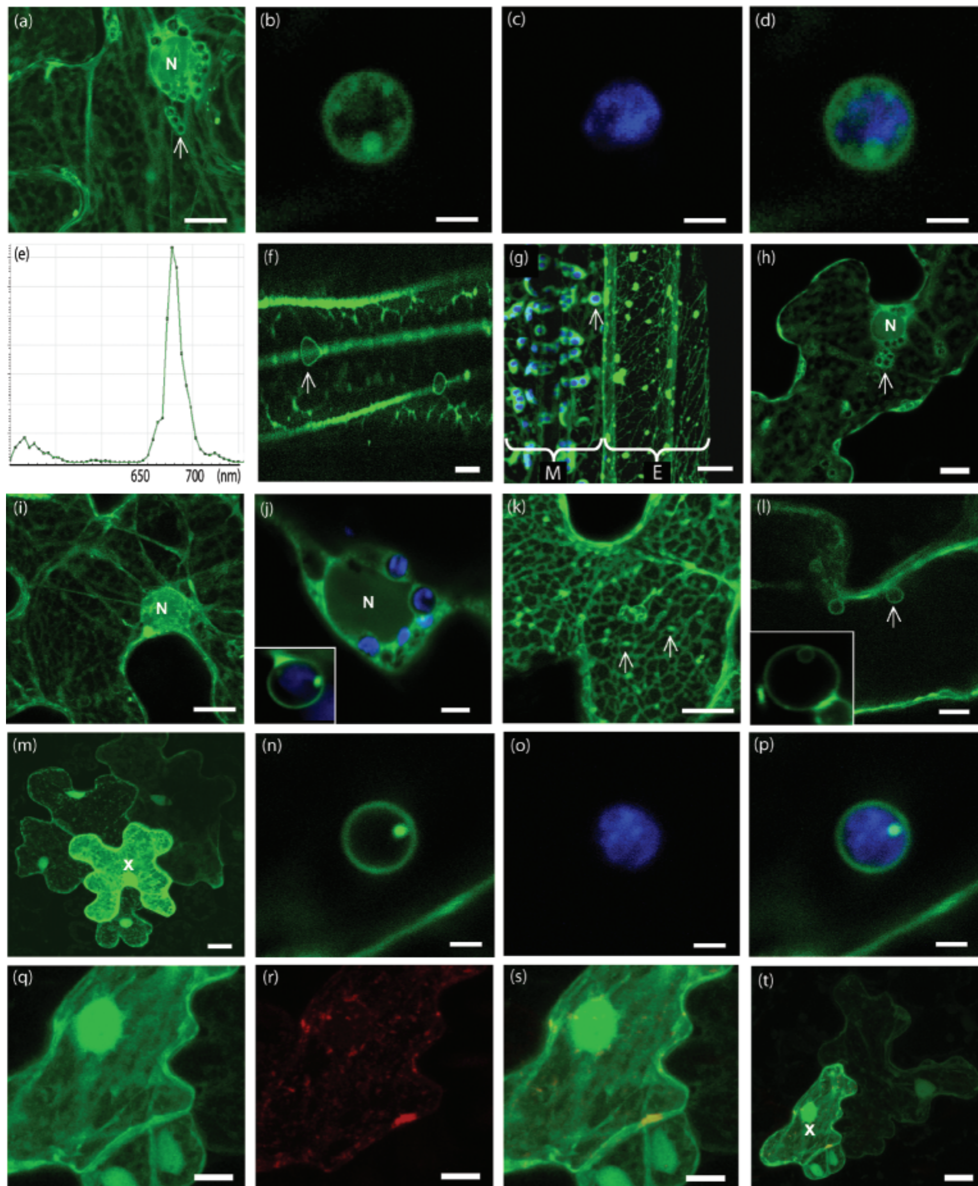
BSMV RNAs α and γ are required exclusively for BSMV replication (Jackson *et al.*, 1991). In our experiments, when RNA β (which encodes the CP and TGB) was omitted from the reporter-clone plasmid preparations bombarded to *N. benthamiana* leaves, BSMV infection was confined to single epidermal cells. In these cells, the subcellular localization of GFP–2A–γb was identical to that observed when all three RNAs were present. Namely, green fluorescence was predominantly cytosolic and there was an association with chloroplasts as described above (arrow in Fig. 2h).

Transiently expressed 35S–GFP–γb protein, when expressed alone, remained cytosolic, but localized to the chloroplasts in the presence of viral RNAs α and γ

Plasmids expressing BSMV GFP–γb or mRFP–γb under the control of the 35S promoter were expressed transiently in epidermal cells of *N. benthamiana*. In these experiments, the fluorescence was mainly cytosolic and small aggregates were sometimes observed (Fig. 2i); there was no obvious accumulation of fluorescence in spots at the periphery, typical of an association with PD, and fluorescence was never observed associated with vesicle or chloroplast membranes. However, when either 35S–mRFP–γb or 35S–GFP–γb was co-bombarded to cells together with BSMV RNAs α and γ, the fusion proteins were associated with most chloroplasts in almost every cell examined (fluorescence was seen in the envelopes and discrete internal spots) and the localizations were indistinguishable from those obtained when the fusion proteins were expressed from the viral vector (Fig. 2j). In contrast, in experiments where 35S–GFP–γb was expressed with a single RNA species, green fluorescence was seen only occasionally in chloroplasts of two of 42 cells when the fusion protein was expressed together with RNA α and in four of 24 cells when expressed together with RNA γ (totals from three independent experiments).

35S–GFP–TGB2 localized to ER membranes, punctate spots and vesicles when expressed alone, but was recruited to chloroplasts in the presence of BSMV RNAs α and γ

Transient expression of BSMV 35S–GFP–TGB2 in *N. benthamiana* epidermal cells showed early association of GFP with ER membranes and small, punctate spots (granules) of green fluorescence moving on the ER (arrows in Fig. 2k). In some cells, a few pleiomorphic vesicles (approx. 4–8 μm) were observed (arrow in Fig. 2l), but these did not contain chlorophyll. The larger vesicles were more numerous in



cells later in expression (48–60 h post-bombardment) and they sometimes contained internal compartments (Fig. 2l, inset). In addition, some green fluorescence remained in the cytosol; green fluorescence also moved into neighbouring cells, sometimes over several cell boundaries (Fig. 2m).

When BSMV 35S–GFP–TGB2 was expressed with the BSMV RNA α and RNA γ, in addition to the above localizations, green fluorescence was almost always associated with chloroplasts, suggesting that GFP–TGB2 was recruited to chloroplasts in the presence of γb and viral replicase proteins (Fig. 2n–p). In these experiments, as before, green fluorescence moved into neighbouring cells. In contrast, and similar to the observations reported in the experiments with 35S–GFP–γb, when 35S–GFP–TGB2 was expressed with individual RNA species, green fluorescence was seen only occasionally in chloroplasts. In three independent experiments, fluorescent fusion proteins were associated with a few chloroplasts in two of 19 cells when expressed with RNA α and one of 14 expressed with RNA γ.

BSMV 35S–GFP–TGB2 and 35S–mRFP–γb did not co-localize when they were expressed transiently in the same cell (Fig. 2q–s). The proteins can be seen in the cytosol and small aggregates of mRFP–γb were seen, but there were no corresponding aggregates of GFP–TGB2 (Fig. 2q, r) and the proteins did not co-localize to membranes or vesicle-like compartments. Moreover, GFP–TGB2 moved into neighbouring cells, whereas mRFP–γb did not (Fig. 2t).

Fig. 2. Expression of BSMV fusion proteins in epidermal cells of *N. benthamiana* or barley. (a–e) Virus reporter clone (RNA α, RNA β and RNA γ–GFP–2A–γb) expressed in *N. benthamiana* epidermal cells. (a) Section of cell; chloroplast arrowed; N, nucleus. Bar, 20 μm. (b–d) Close-up images of vesicle, showing fluorescence as a ring surrounding discrete patches of fluorescence; excitation wavelength 488 nm and emission collected at 500–530 nm (b) or 660–700 nm (c). (d) Images (b) and (c) merged; chlorophyll autofluorescence artificially coloured blue. Bars, 2 μm. (e) Lambda scan (495–755 nm) within the vesicle, showing emission peak at 680 nm (typical of chlorophyll *a*). (f) BSMV clone expressing GFP–2A–γb in barley epidermal cell; arrow points to pleiomorphic vesicle. Bar, 10 μm. (g) BSMV clone expressing GFP–2A–γb in barley epidermal (E) and mesophyll (M) cells; arrow points to chloroplast; chlorophyll autofluorescence artificially coloured blue. Bar, 20 μm. (h) Expression of BSMV clones RNA α and RNA γ–GFP–2A–γb (without RNA β) in *N. benthamiana* epidermal cells, showing green fluorescence localization identical to that shown in (a); arrow points to chloroplast. Bar, 10 μm. (i) Transient expression of 35S–GFP–γb in *N. benthamiana* epidermal cells; green fluorescence remains cytosolic. Bar, 15 μm. (j) Transient expression of 35S–GFP–γb together with RNA α and RNA γ in *N. benthamiana* epidermal cells; green fluorescence is seen in vesicle clusters surrounding nucleus and in vesicles as a ring surrounding discrete patches (inset). Bar, 4 μm. (k) Transient expression of 35S–GFP–TGB2 in *N. benthamiana* epidermal cells, showing labelling of ER network and moving granules (arrowed). Bar, 10 μm. (l) Transient expression of 35S–GFP–TGB2 in *N. benthamiana* epidermal cells, showing GFP in ring-like pleiomorphic vesicles (arrowed). Inset shows vesicle within vesicle. Bar, 10 μm. (m) Transient expression of 35S–GFP–TGB2 in *N. benthamiana* epidermal cells, showing movement of fluorescence from initial cell (X) over several cell boundaries. Bar, 10 μm. (n–p) Transient expression of 35S–GFP–TGB2 together with RNA α and RNA γ; excitation was at 488 nm and emission was collected at 500–530 nm (n) or 660–700 nm (o). (p) Images (n) and (o) merged; chlorophyll autofluorescence artificially coloured blue. Green fluorescence is localized to chlorophyll-containing vesicles. Bar, 2 μm. (q–t) Transient expression of 35S–GFP–TGB2 co-bombarded with 35S–RFP–γb in *N. benthamiana* epidermal cells. (q) Green channel; (r) red channel; (s) merged. Bar, 5 μm. (t) Merged image of red and green channels, showing movement of GFP fluorescence from initial cell X. Bar, 10 μm.

EM of thin sections of BSMV-infected *N. benthamiana* revealed virus-like particles associated with abnormal chloroplasts

EM of thin sections of BSMV reporter clone (RNA α, RNA β and RNA γ–GFP–2A–γb)-infected *N. benthamiana* and barley leaves showed that chloroplasts in infected cells were abnormally rounded or distorted and the grana stacks were often disrupted (Fig. 3a, c, e). The rounded chloroplasts were approximately 4 μm in diameter and many contained membrane-bound cytoplasmic inclusions. Sometimes, the chloroplasts contained inclusions that protruded into the cytoplasm (arrow in Fig. 3b; Fig. 3e). In tissue treated with osmium tetroxide to enhance membrane contrast (Hawes & Satiat-Jeunemaitre, 2001), some of the cytoplasmic inclusions were seen to be surrounded by a double membrane (Fig. 3c), suggesting that they may be formed by invagination of the chloroplast envelope. Balloon-like cytoplasmic inclusions were also seen in chloroplasts that had maintained an ovoid shape. Some small invaginations can also be seen in the outer envelope membrane in Fig. 3(d). Tubular, rod-shaped virus-like particles were found within the cytoplasmic inclusions (Fig. 3c; insert in Fig. 3e), in the cytoplasm surrounding the chloroplasts (insert in Fig. 3a), appressed against the outer chloroplast membrane (insert in Fig. 3f) and in paracrystalline arrays within the chloroplast stroma (insert in Fig. 3b). There were many rounded and distorted chloroplasts in BSMV-infected cells in both barley and *N. benthamiana*; such abnormalities were not observed in chloroplasts in uninfected tissue (data not shown). As seen in the confocal images, the chloroplasts in infected cells were found to cluster together around the nucleus (Fig. 3b).

Viral RNA, γb and CP were detected in plastid preparations

Preparations of chloroplasts were isolated from whole-leaf tissue following standard methods (Robinson, 1994); in these experiments, we cannot rule out the possibility that the preparations also contained other kinds of plastids, so, in this section, we use the term plastid rather than chloroplast. Enriched plastid preparations were obtained from barley-leaf tissue infected with the BSMV reporter clone (RNA α, RNA β and RNA γ–GFP–2A–γb). Green fluorescent, chlorophyll-containing plastids were seen when the preparations were examined by CLSM (data not shown). Western blots of the plastid preparations revealed the presence of BSMV CP, GFP–2A–γb and γb proteins (Fig. 4), although there was less γb in plastids than in whole-leaf extract. RT-PCR analysis confirmed the presence of viral RNA in the preparations. Positive-strand viral RNA γ was detected at a comparable level in both total RNA originating from either whole-leaf extract or from plastid preparations from either barley (Fig. 4) or *N. benthamiana* (data not shown) infected leaves.

DISCUSSION

BSMV γb protein is a 17 kDa, cysteine-rich, multifunctional protein that has been shown to play a role in virus pathogenicity (Donald & Jackson, 1994), accumulation of CP and TGB1 proteins (Petty *et al.*, 1990) and suppression of RNA silencing (Yelina *et al.*, 2002; Bragg & Jackson, 2004). In previous work using a BSMV γb–GFP reporter clone, it was shown that fusion of GFP to the C terminus does not affect γb protein function substantially (Lawrence & Jackson,

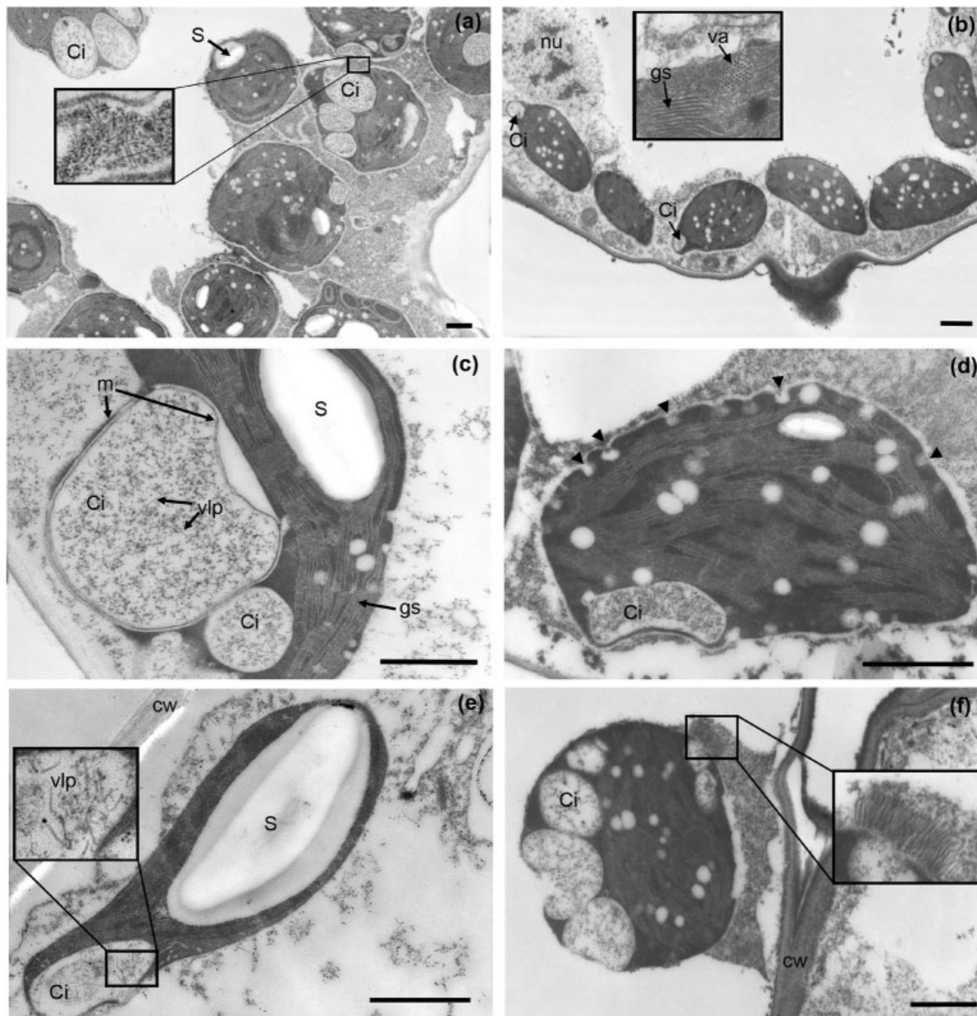


Fig. 3. EM images of thin sections of *N. benthamiana* (a, c, e) and *H. vulgare* 'Black Hulless' (b, d, f) leaves infected with BSMV containing the reporter clone GFP–2A–γb. Chloroplasts of infected cells were abnormally rounded (a) and found to cluster near the nucleus (b). Deformed chloroplasts containing characteristic membrane-bound cytoplasmic inclusions caused by BSMV infection are shown in (c) (post-fixed with osmium tretroxide) and (d) (not post-fixed with osmium tetroxide). Cytoplasmic invaginations at the periphery of the chloroplast are highlighted by arrowheads in (d). Virus-like particles were found in the cytoplasm abutting chloroplasts [insert in (a)], within the cytoplasmic inclusions (c, e), appressed against the outer chloroplast membrane (f) and within the chloroplast stroma [insert in (b)]. S, Starch granule; Ci, cytoplasmic inclusion/invagination; vlp, virus-like particles; m, membrane; nu, nucleus; cw, cell wall; gs, grana stack; va, paracrystalline virus aggregate. Bars, 1 μm.

2001b). In this study, we found that expression of BSMV reporter clones containing GFP-tagged γb revealed both a cytosolic localization and an association with abnormal chloroplasts in infected cells of both monocot and dicot hosts. Green fluorescence was associated with the chloroplast envelope and discrete patches inside. We found similar results irrespective of whether GFP was fused to the N or C terminus of γb. Furthermore, γb targeting to chloroplasts was independent of the presence of CP or TGB proteins, but it did require the presence of BSMV RNA α or RNA γ, and recruitment was more efficient when RNA α and RNA γ were both present. Transient expression of GFP–γb fusion protein alone from the 35S promoter resulted in cytosolic localization of green fluorescence.

Fluorescence of transiently expressed BSMV 35S–GFP–TGB2 in epidermal cells showed an initial localization to ER membranes and ER-associated motile granules similar to those seen for PMTV TGB2 (Haupt *et al.*, 2005). BSMV 35S–GFP–TGB2 was also observed in the membranes of pleiomorphic vesicle-like compartments and green fluorescence moved into adjacent cells over several cell boundaries. Furthermore, like 35S–GFP–γb, BSMV 35S–GFP–TGB2 was targeted to chloroplasts, but only when co-bombarded with

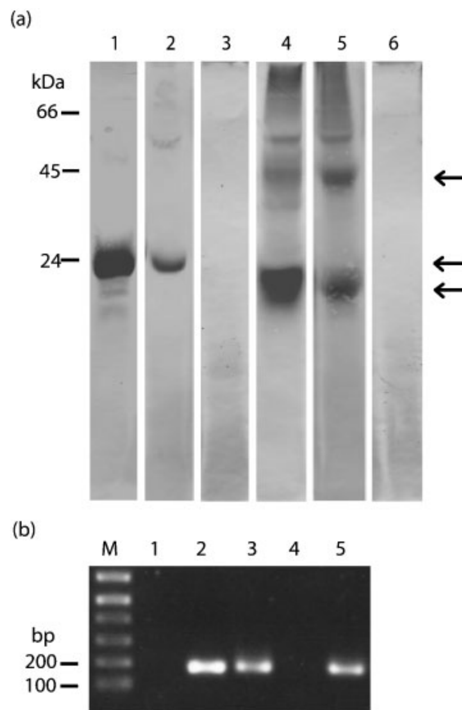


Fig. 4. Detection of BSMV genomic RNA γ and virus-encoded proteins in plastid preparations from barley leaves infected with BSMV reporter clone GFP–2A–γb. (a) Western blots of whole-leaf extracts from infected plants (lanes 1 and 4), plastid preparations from virus-infected leaves (lanes 2 and 5) and non-infected leaves (lanes 3 and 6) reacted with anti-CP (lanes 1–3) or anti-γb (lanes 4–6); arrows indicate positions of GFP–2A–γb, CP and γb proteins in descending order; the positions of the molecular mass markers are indicated on the left. (b) RT-PCR from whole-leaf extracts of infected plants (lane 3) and plastid preparations from either uninfected leaves (lane 4) or from BSMV-infected leaves (lane 5) were reverse-transcribed by using γb-specific antisense primers followed by PCRs using nested primers for 30 cycles to amplify a 200 nt BSMV γ cDNA fragment. Non-template control, plasmid positive control and molecular markers are in lanes 1, 2 and M, respectively. Similar quantities of PCR product were amplified from total barley-leaf extract and chloroplasts.

RNA α or RNA γ, and targeting was more efficient when both RNAs were present. Localization of BSMV 35S–GFP–TGB2 revealed some similarities and differences compared with that of PMTV TGB2. Association with ER membranes and motile granules is similar to that observed for GFP-tagged PMTV TGB2, but PMTV TGB2 also localized to the membranes of subcellular vesicle populations derived from the plasma membrane (Haupt *et al.*, 2005) and to the chloroplast envelope when expressed transiently in the absence of virus RNA (Torrance *et al.*, 2006; G. Cowan & L. Torrance, unpublished results). Moreover, fluorescently tagged PMTV TGB2 never moved out of the cell (Haupt *et al.*, 2005). These results indicate that there are subtle differences in function of the hordei-like TGB2 proteins.

Previous results with the BSMV reporter clone containing RNA γ–γb–GFP using fluorescence microscopy showed that γb–GFP fluorescence remained cytosolic in infected cells and that in barley, surprisingly, GFP expression was greater in mesophyll cells and was not detected readily in epidermal cells, whereas in *N. benthamiana*, GFP fluorescence was detected readily in epidermal cells (Lawrence & Jackson, 2001b). Our results using CLSM help to explain these findings. Fluorescence was probably detected less readily in barley epidermal cells because they do not contain chloroplasts, but barley mesophyll cells contain large numbers of chloroplasts with high levels of γb–GFP fluorescence. The Western blots of plastid preparations showed that although γb was detected in plastids, the relative amounts were lower than those in whole-leaf extracts. Previously, detailed subcellular-fractionation studies of infected barley leaves showed that γb was present mainly in the soluble (cytosolic) fraction, with much lower amounts in the P1 (nuclei and chloroplasts) and P30 (membrane) fractions (Donald *et al.*, 1993). Taken together with the CLSM images showing high levels of cytosolic localization, the results suggest that γb is predominantly cytosolic, with a small proportion targeted to plastids.

The EM images of abnormally rounded chloroplasts with cytoplasmic invaginations and protrusions and their association with virus-like particles obtained by using the BSMV reporter clone are very similar to images of thin sections of BSMV-infected barley published by Carroll (1970). The cytoplasmic invaginations in the chloroplasts and vesicles seen between the inner and outer envelope membranes correspond remarkably to the fluorescence in the chloroplast envelope and discrete patches within that were seen in our CLSM images. Previous EM immunogold-labelling studies detected BSMV CP and particles within cytoplasmic invaginations, on vesiculated membranes of proplastids and associated with the chloroplast envelope (Lin & Langenberg, 1984). Interestingly, these authors also found BSMV CP associated with the plasmalemma, ER and nuclei, but not with mitochondrial membranes, Golgi apparatus or peroxisomes (Lin & Langenberg, 1984). Antibodies against poly(I) : poly(C) were used in immunogold-labelling experiments to detect double-stranded RNA in proplastids of BSMV-infected wheat-root tips (Lin & Langenberg, 1985) and the authors concluded that proplastids play a role in BSMV replication. Replication of *Turnip yellow mosaic virus* is also associated with small, peripheral vesicles formed from invaginations at the chloroplast envelope, and immunogold labelling of thin sections by using RNA-dependent RNA polymerase (RdRp)-specific antiserum showed viral RdRp associated with them at the periphery of the chloroplast (Prod'homme *et al.*, 2001). In our experiments, virus-like particles and paracrystalline aggregates were seen within chloroplast cytoplasmic invaginations and the stroma, and CP, γb and viral RNA were detected in leaf-plastid

preparations, supporting the conclusion that chloroplasts are sites of virus replication.

Positive-sense ssRNA viruses must have a means of regulating the use of the viral genome as a template for replication from the processes of translation and transport. Examples of strategies to separate these competing processes are found in viruses such as BMV and *Poliovirus* (Ahlquist *et al.*, 2003). BMV RNAs are recruited from translation to replication through the interaction of *cis*-acting recognition elements in the RNA with the 1a protein (a helicase-like replication factor homologous to the αa protein of BSMV) into vesicles (spherules) that form in the ER in BMV-infected cells (Ahlquist *et al.*, 2003). Such membrane structures are likely to be the functional equivalent of the chloroplast invaginations we observed in this work. It is possible that recruitment of BSMV RNA to plastids could be a mechanism to achieve the spatial separation of these phases and to isolate the RNA template for genome replication and production of virus particles. The fact that viral RNAs α and γ (encoding the replicase proteins and γb) are sufficient to recruit γb and TGB2 to chloroplasts suggests that the replicase proteins (αa or γa) may assist in translocation of the viral RNAs to this compartment, and also that there are protein–protein or protein–RNA interactions between αa and/or γa and γb or TGB2. The fact that BSMV can replicate its viral RNA and move systemically in spite of reduced accumulation levels of viral RNAs in the absence of γb suggests some degree of functional redundancy between these proteins, at least in leaf tissues. It could be that γb has a regulatory role in the complex, e.g. downregulating template translation, or that it may be required later in the infection cycle of BSMV to promote pollen and seed invasion and dissemination of viral progeny. Indeed, seed-transmission determinants of BSMV were identified in the γb gene (Edwards, 1995), where its role as silencing suppressor maybe an important feature for meristem- and seed-tissue invasion.

Edwards (1995) showed, by using pseudorecombinants between RNAs of BSMV strains ND18 (readily seed-transmitted) and CV17 (not readily seed-ransmitted), that seed transmissibility was determined largely by RNA γ, with major determinants located in the 5′ untranslated leader sequence, a 370 nt repeat sequence in the γa gene and the γb gene, but that RNAs α and β also played a role. In our experiments, RNA γ was sufficient to target γb–GFP to plastids, but the efficiency was increased when RNA α was also present. Our data suggest a mechanistic explanation for the results of Edwards (1995); viral RNA γ and γb protein are needed in the establishment of plastid-localized virus replication complexes and, if such an association is not established, then virus spread into developing embryos through chloroplast inheritance from maternal tissue would not occur.

The homologous, cysteine-rich γb protein of *Poa semilatent virus* was found to be targeted to peroxisomes (Yelina *et al.*, 2005) and targeting was attributed to the presence of an SKL motif at the C terminus of the protein. This motif is also thought to be responsible for targeting the cysteine-rich P15 protein of *Peanut clump virus* (Yelina *et al.*, 2005). However, only two of the four sequenced BSMV strains have γb proteins with SKL motifs, and ND18 (the strain used to derive the reporter clones in our experiments) does not; the sequence terminates with SK, which probably explains why we did not observe peroxisomal targeting in our experiments.

The cysteine-rich γb protein is a pathogenicity determinant and, although it is not essential for systemic infection in the ND18 strain, deletion mutants show milder symptoms with decreased accumulation of viral RNAs α, β and γ and proteins CP and TGB1 (Petty *et al.*, 1990). At the N terminus of the γb protein, there are two clusters of cysteine residues (C1 and C2) separated by a short stretch of basic amino acids, and mutation of cysteine residues in these clusters produces distinctive phenotypes (Donald & Jackson, 1994). For example, specific mutations in the C1 motif produced severe bleached symptoms with elevated levels of the γb protein, whereas three mutations in the C2 motif and one in the basic region produced milder symptoms with a patchy mosaic phenotype (symptoms similar to those obtained when the γb gene was deleted from the clone), accompanied by decreased amounts of detectable CP and TGB1. It has also been shown that the basic region is responsible for RNA binding, whereas the C1 and C2 regions bind zinc (Donald & Jackson, 1996; Bragg *et al.*, 2004). Moreover, the γb sequences of CV17 and ND18 differ in only four amino acids, two of which are located in the C1 and C2 regions and are correlated with changes in seed transmissibility (Edwards, 1995). There is no obvious correlation between symptom phenotype and individual mutations that alter γb RNA- or zinc-binding activity; it is possible that some individual mutations are compensated by amino acids at other positions or that the effects are obscured, e.g. because of a requirement for interaction with specific host factors. However, what is clear is that these regions are important in the function of γb and its effects on pathogenicity. Taken together with our results, it seems possible that domains within the C1–basic–C2 regions that affect the ability of γb to bind RNA and zinc and possibly to interact with other virus proteins and host factors may be an important determinant in the recruitment and establishment of virus replication complexes in plastids and/or in the regulation of replication processes. Deletions or point mutations in γb that interfere with this function may result in the observed milder symptoms and decreased accumulation of virus RNA and virus-encoded proteins. Conversely, mutations leading to severe symptoms may be due to higher levels of virus infection, inducing disruption of plastids or affecting chloroplast development.

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