Running title: Phosphorylation of TGBp3 in Potato mop-top virus

Tyrosine Phosphorylation of the Triple Gene Block Protein 3 Regulates Cell-to-Cell Movement and Protein Interactions of Potato mop top virus

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Functions of viral proteins can be regulated through phosphorylation by serine/threonine kinases in plants but little is known about involvement of tyrosine kinases in plant virus infection. In this study, TGBp3, one of the three movement proteins encoded by a triple gene block (TGB) of Potato mop-top virus (PMTV), was detected for the first time in PMTV-infected plants and found to be tyrosine-phosphorylated. Phosphorylation sites (Tyr87-89 and Tyr120) were located in two amino acid motifs conserved in the TGB-containing, rod-shaped plant viruses. Substitution of these tyrosine residues in both motifs was needed to abolish tyrosine phosphorylation of TGBp3. Substitution of Tyr87-89 with alanine residues enhanced the interaction between TGBp3 and TGB protein 2 (TGBp2) and inhibited cell-to-cell movement of PMTV. On the other hand, substitution of Tyr120 with alanine resulted in no alteration in the interaction of TGBp3 with TGBp2, but the mutant virus was not infectious. The results suggest that tyrosine phosphorylation is a mechanism regulating the functions of plant virus movement proteins.
Plant viruses encode movement proteins (MPs) to facilitate intra- and intercellular movement of viral genomes to and through plasmodesmata by recruiting the host trafficking systems (1, 2, 3, 4, 5). Much research has focused on the mechanisms regulated by MPs, but less is known about regulation of MP activities. Protein phosphorylation causes reversible posttranslational modification that plays a fundamental role in the regulation of many cellular processes in eukaryotic cells, including altering protein function, interactions, stability, or subcellular location (6). However, the regulation of plant virus movement by phosphorylation has been studied in only a few taxa.

Phosphorylation of MPs by cellular serine (Ser)/threonine (Thr) kinases can either enhance or inhibit virus movement, indicating the importance of phosphorylation in the virus infection cycle. For example, phosphorylation of the 30K MP of Tobacco mosaic virus (TMV, genus Tobamovirus) is needed for MP-mediated gating of plasmodesmata and mediation of viral cell-to-cell movement (7, 8, 9). In another member of Tobamovirus (Tomato mosaic virus), phosphorylation enhances MP stability and controls its intracellular localization (10, 11).

Phosphorylation of the coat protein of Potato virus A (PVA, genus Potyvirus) by the host kinase CKII in tobacco (Nicotiana tabacum) cells inhibits RNA binding by the coat protein as well as cell-to-cell movement of PVA (12, 13). In the bipartite viruses of the family Geminiviridae, the phosphorylated nuclear shuttle protein facilitates intracellular transport of viral DNA from the nucleus to the cytoplasm and promotes cell-to-cell movement of the virus in concert with MPs (14).

Genomes of many plant viruses with rod-shaped particles (family Virgaviridae and genus Benyvirus) or filamentous particles (family Alphaflexiviridae) contain three MP genes organized
as a ‘triple gene block’ (TGB) (15). However, little is known about phosphorylation of the three MPs. The TGB protein 1 (TGBp1) of *Potato virus X* (genus *Potexvirus*) is phosphorylated in tobacco plants, but the functional role of the phosphorylation is not known (16).

*Potato mop-top virus* (PMTV; genus *Pomovirus; Virgaviridae*) is a TGB-containing virus that causes yield losses in potato production by inducing necrotic arcs in the infected tubers that precludes their marketability (17). PMTV has a tripartite, (+)ssRNA genome consisting of RNA1 (6.0 kb), RNA2 (3.2 kb), and RNA3 (2.9 kb) segments (18, 19). RNA3 contains four open reading frames predicted to encode the TGB proteins TGBp1 (51K), TGBp2 (13K) and TGBp3 (21K) and an 8K cystein-rich protein (19, 20, 21) (Fig. 1A). Co-expression of TGB proteins from plant expression vectors in the same cell has revealed their coordinated functions, suggesting a model in which TGBp3 and TGBp2 mediate the transport of a TGBp1-containing ribonucleoprotein complex to and through the plasmodesmata, whereas TGBp2 and TGBp3 are not transported to the adjacent cell (22). These roles of TGBp3 and TGBp2 are consistent with their ability to increase the size exclusion limit of plasmodesmata (23). Yeast two-hybrid assays have shown that TGBp3 and TGBp2 self-interact and interact with each other (24, 25), which is important for TGB functions because disruption of the TGBp2-TGBp3 interaction inhibits the movement of *Barley stripe mosaic virus* (BSMV, genus *Hordeivirus*) (26, 27). Previous studies suggest that TGB proteins must be expressed at suitable relative molarities for viral movement to occur. For example, optimal relative expression ratio of TGBp2 and TGBp3 is 10:1 in BSMV and PMTV (25, 28), and TGBp2 and TGBp3 should be expressed at 10- and 100-fold lower levels, respectively, than TGBp1 (27, 29, 30). The studies on PMTV TGBp3 have been done by producing the protein from a plant expression vector or heterologous virus (e.g., 23, 24, 25).

Indeed, the TGBp3 proteins present in infected plant tissues are extremely difficult to detect due

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to a low titer (26, 29, 31, 32) and previous attempts to detect and localize the PMTV TGBp3 in PMTV-infected plants have been unsuccessful (24). Hence, unequivocal evidence that TGBp3 of PMTV is expressed during virus infection remains to be obtained.

The TGBp3 in hordei-like viruses contains a conserved YQDLN motif in the central part of the protein (33). The YQDLN motif in the PMTV TGBp3 serves a critical role during infection of plants. When the motif is mutated to GQDGN, TGBp3 is no longer targeted to plasmodesmata and is impaired in its ability to gate plasmodesmata open (23). Hence, tyrosine (Tyr) at position 89 appears to be crucial for viral cell-to-cell movement (25).

Little is known about phosphorylation of TGB proteins. It is also unclear whether tyrosine kinases participate in phosphorylation of MPs or other viral proteins in plants. Because the Tyr-containing motif in PMTV TGBp3 is important for viral movement, the aim of this study was to examine possible tyrosine phosphorylation of TGBp3 to gain further insight into the functions and regulation of TGBp3 activity in PMTV.

MATERIALS AND METHODS

Cloning and mutagenesis of DNA

The plasmid pPMTV3 contains a full-length cDNA clone of PMTV RNA3 that can be used to generate RNA3 transcripts in vitro and generate infectious PMTV when co-inoculated with the RNA1 and RNA2 transcripts to plants (34).

The putative phosphotyrosine sites in PMTV TGBp3 were predicted using NetPhos 2 and Scansite. The NetPhos 2 algorithm is a neural network method with a false-positive prediction rate of 0–26% for tyrosine (35). Scansite predicts target motifs for different kinases using a
positional selectivity matrix based on peptide library screening (36). Searches using Scansite applied a high level of stringency to identify the strongest motif matches.

To produce the various constructs described below, pPMTV3 was subjected to PCR-based modification and site-directed mutagenesis using the high-fidelity Phusion DNA polymerase (Finnzymes, Espoo, Finland) as described (37). Tyr-to-alanine (Ala) substitutions were introduced in the residues Tyr87, Tyr88, and Tyr89 (construct pPMTV321K87–89A), or Tyr120 (pPMTV321K120A) (Figs. 1A, B). Furthermore, all mutations were combined in the construct pPMTV321K87–89A/120A (Fig. 1B). The primers used to prepare the aforementioned and other constructs in this study are available upon request.

The sequence encoding the Myc epitope (EQKLISEEDL) was added to the 3'-end of TGBp3 (pPMTV321K-Myc) (Fig. 1A). To produce GFP-fusion constructs of pPMTV3, an NcoI site was created at the 5'-end of the TGBp1 gene using PCR-based mutagenesis. The GFP coding sequence was amplified from vPVA-GFP (38) and subsequently inserted at the 5'-end of the 51K gene of wild-type (wt) or mutant pPMTV3 constructs (Fig. 1A) to obtain GFP-pPMTV3, GFP-pPMTV321K87–89A, GFP-pPMTV321K120A, and GFP-pPMTV321K87–89A/120A.

To express PMTV TGBp3 in fusion with glutathione S-transferase (GST) (GST-21K), 21K was cloned in the pGEX 6P-1 expression vector (Amersham Biosciences, Piscataway, NJ, USA) to obtain construct 21KpGEX6P-1. For expression in plants under the Cauliflower mosaic virus 35S promoter, 21K-myc and the 8K gene of PMTV were cloned in the binary vector pLH (39). Cloning was done according to standard molecular biology protocols (40). Construct p21K_Myc_pLH was used as the parental plasmid for site-directed mutagenesis to obtain the mutated genes for expression of 21K87–89A, 21K120A and 21K87–89A/120A as Myc-tagged proteins from binary vectors in plants.
For yeast two-hybrid system (YTHS) analysis, the mutated TGBp3 gene sequences encoding the 21K87–89A, 21K120A, and 21K87–89A/120A proteins were obtained by PCR from parental plasmids pPMTV321K87–89A, pPMTV321K120A, and pPMTV321K87–89A/120A, respectively, using Dynazyme II DNA polymerase (Finnzymes) according to manufacturer’s instructions. The genes for 13K and 21K proteins were amplified by PCR as described above using the wt pPMTV3 plasmid as the template. The obtained PCR products were cloned into pGEM-T (Promega, Madison, WI, USA), and plasmid DNA was then prepared and cleaved with appropriate restriction enzymes for subsequent cloning into similarly cleaved pGADT7 and pGBK7 vectors (Clontech, Mountain View, CA, USA). All the constructs were verified by sequencing.

Expression and purification of recombinant proteins

Escherichia coli strain BL21 was transformed with p21KpGEX6P-1 and grown to an optical density of 0.6 at 600 nm in Luria broth (Sigma-Aldrich, St. Louis, MO, USA). Protein expression was induced by addition of isopropyl β-D-1-thiogalactopyranoside (Promega) to a final concentration of 0.1 mM, and the cells were grown for 3 h at 30°C and collected by centrifugation (5000 × g for 10 min). The protein was purified under native conditions on glutathione-Sepharose 4B (Amersham Biosciences) according to the manufacturer’s protocols, with some modifications (41). Polyclonal antiserum against 21K was produced by immunization of two rabbits with purified GST-21K. The IgG fraction was purified on protein A–Sepharose CL-4B (GE Healthcare, Waukesha, WI, USA) according to the manufacturer’s instructions.
**In vitro and in vivo kinase assays and western blotting**

Leaf tissue from full-grown leaves of 5-wk old *Nicotiana benthamiana* plants (see below) was homogenized in 20 mM Hepes-KOH buffer (pH 7.4) with a pestle and a mortar prechilled to 4°C. The leaf extract (total protein concentration 10 µg/ml) as the source of plant kinase activity and 10X kinase buffer were mixed in 10:1 ratio (final concentration 20 mM Hepes-KOH, pH 7.4, 10 mM MgCl₂, 50 mM NaCl, 1 mM dithiothreitol, 10 mM MnCl₂). Purified GST-21K protein (200 ng) was added to an aliquot (20 µl) of the solution and incubated in the presence of 10 µCi [$\gamma$-$^{33}$P]ATP (Amersham Biosciences) for 45 min at 37°C. Reaction was terminated by adding SDS-PAGE sample buffer, followed immediately by boiling for 5 min. The samples were analyzed by SDS-PAGE (12.5% w/v acrylamide) and subjected to autoradiography.

In another experiment, 500 ng of purified GST-21K was incubated as above in the presence of 1 mM unlabeled ATP. Subsequently, half of the probe was left untreated or treated with λ-protein phosphatase (New England Biolabs, Ipswich, MA, USA) according to the manufacturer’s protocols. The samples were subjected to SDS-PAGE (12.5% w/v acrylamide) and western blotting (see below).

In a third type of experiment, the Myc-tagged wt 21K protein (21K-myc) and mutants were expressed in leaves of *N. benthamiana* by agroinfiltration (see below). Agroinfiltrated leaf tissue was sampled 3 days post-infiltration, ground in SDS-PAGE sample buffer at 1:3 ratio (w/v) at room temperature and heated to 95°C for 5 min. Aliquots of 20 µl were analyzed by SDS-PAGE (12.5% w/v acrylamide), after which proteins were transferred onto a polyvinylidene fluoride membrane (Amersham Biosciences). Western blotting was done using a specific anti-phosphotyrosine (α-p-Y) (clone 4G-10 Platinum; Upstate, Millipore, USA) or anti-Myc monoclonal antibody (α-myc) (9E10; Santa Cruz Biotechnology, Santa Cruz, CA, USA), or
polyclonal antibodies to the PMTV 21K protein (prepared in this study). Polyclonal rabbit anti-
mouse immunoglobulins (IgG) conjugated with horse radish peroxidase (HRP) (Dako Denmark A/S, Glostrup, Denmark) and HRP-conjugated anti-rabbit donkey IgG (GE Healthcare, Little Chalfont, UK) were used as secondary antibodies. Each membrane was developed with the SuperSignal West Femto Chemiluminescence detection system (Thermo Scientific, Rockford, IL, USA).

**Yeast two-hybrid system (YTHS) assay**

Yeast competent cells were prepared and transformed using MATCHMAKER GAL4 Two-Hybrid System 3 (Clontech) with yeast strain AH109 and a small transformation scale according to manufacturer’s instructions. Co-transformations were made with different combinations of pGADT7 activation domain (AD) and pGBK7 DNA-binding domain (BD) constructs. Co-transformation of pGBK7-53 (Clontech) with pGADT7-T (Clontech) was used as positive interaction control and pGBK7-Lam (Clontech) with pGADT7-T as a negative interaction control. To select for co-transformants, the cells were plated on supplement dropout medium lacking leucine and tryptophan (SD/−Leu/−Trp) and grown at 30ºC for approximately 36 h. The colonies were then replated on SD/−Adenine (Ade)/−Histidine (His)/−Leu/−Trp to check for interaction between the two-hybrid proteins as indicated by growth on the selection plate after a one-week incubation at 30ºC.

To test the strength of the interactions, yeast strain Y187 was co-transformed with the above-described constructs, and the pellet X-gal β-galactosidase assay was performed and analyzed as described (42). The intensity of the blue color, caused by cleavage of X-gal by β-galactosidase (β-gal) activity, was determined with ImageJ software (43). These intensities were...
used to calculate β-gal activity relative to the positive control that was arbitrarily set to 100%.

Expression of the fusion proteins in yeast was verified by western blot analysis using AD and BD specific MAbs (Clontech) as described (44).

### Plant materials and growth conditions

*N. benthamiana* plants were grown in a controlled growth room at 20°C with a 16-h photoperiod. Five-week-old plants were used in the experiments. For inoculations, the plants were moved to a growth chamber (16°C, 16-h photoperiod) and kept in the dark overnight prior to inoculations. Infiltrated and bombarded plants were kept in the growth room during the experiments. All plants were fertilized weekly with a 1% (w/v) solution of N:P:K (16:9:22) fertilizer (Yara, Espoo, Finland).

### Agroinfiltration

The binary vectors for expression of PMTV proteins were introduced by electroporation into *Agrobacterium tumefaciens* strain C58C1 containing Ti plasmid pGV3850 (45). The transformations were verified with PCR designed to target the pLH vector from both sides of the cloning site. For agroinfiltration, the transformed cells were grown overnight at 28°C with shaking. The cells were collected by centrifugation and resuspended in induction medium (10 mM MgCl$_2$, 10 mM MES, 200 μM acetosyringone) at a final OD$_{600}$ of 1.0 and induced at room temperature for 3 h. Two or three fully expanded leaves of *N. benthamiana* plants were used for agroinfiltration. After 3 days, the agroinfiltrated leaves were collected and analyzed.
Virus inoculation and detection

RNA transcripts were synthesized from PMTV cDNA constructs as described (34). Linearized cDNA (2 µg) was used in each transcription reaction. The fully expanded leaves of N. benthamiana plants were mechanically inoculated with RNAs in GKP buffer (46) consisting of 12.5 mM glycine, 7.5 mM K$_2$HPO$_4$ (pH 9.2), 0.25% (w/v) bentonite (Sigma-Aldrich) and 0.25% (w/v) celite (Fluka Chemie, Buchs, Switzerland). Inoculation was done with a mixture containing wt or mutant RNA1:RNA2:RNA3 (1:1:2). Control plants were mock inoculated with the GKP buffer.

Inoculated and upper leaves were collected at 18 days post-inoculation (dpi). Samples were tested for the presence of PMTV by double-antibody sandwich ELISA (DAS-ELISA) using a MAb specific to PMTV coat protein (Science and Advice for Scottish Agriculture SASA, Edinburgh, UK) as described (47) and/or reverse transcription–PCR (RT-PCR). In a parallel experiment, leaves were collected at 3, 7, 10, and 14 dpi, and the samples were analyzed by RT-PCR. For RT-PCR, RNA was extracted using Trizol-like reagent (48). RNA samples were treated with RQ1 RNase-free DNase (Promega) at 37°C for 30 min. cDNA was synthesized with Moloney murine leukemia virus reverse transcriptase (Promega) according to the manufacturer’s instructions. To initiate cDNA synthesis random hexamers were used. RT-PCR was carried out using Phusion DNA polymerase (Finnzymes) following the manufacturer’s instructions.

In another experiment, plants were co-inoculated with wt RNA1, RNA2 and wt or mutant GFP-RNA3. Infections were followed daily up to 18 dpi using a hand-held UV light (B-100 AP; UVP, Upland, CA). To study the infection at the single-cell level, plants were inoculated by bombardment with gold particles coated with transcribed RNAs using a HandyGun as described (49). Each single bombardment contained 0.1 mg gold particles (1 µm diameter; Bio-Rad,
Hercules, CA, USA) coated with RNA transcribed from 50 ng pPMTV1, 50 ng pPMTV2, and 100 ng of wt or mutant pPMTV3 plasmids. Leaves were analyzed at different time points up to 7 dpi.

Confocal microscopy

Leaf tissue from the edges of the bombardment site was fixed to a microscope slide using coverslips and tape, and mounted in water. Pictures were taken with a Leica TCS SP5II HCS A confocal microscope using an HC PL APO 10×/0.4 objective. GFP was visualized using argon laser excitation at 488 nm and an acquisition window of 500–552 nm. LAS AF Lite software package (Leica Microsystems GmbH, Wetzlar, Germany) was used to construct the images from serial optical sections and for image processing.

RESULTS

Detection of TGBp3 in PMTV-infected plant tissues

Extracts from *N. benthamiana* leaves infected with PMTV or agroinfiltrated for 35S promoter–driven TGBp3 expression were tested by western blotting using polyclonal antibodies raised to PMTV TGBp3 (21K protein) in this study. The antibodies detected a protein with electrophoretic mobility corresponding to TGBp3 in the agroinfiltrated leaf tissues over-expressing TGBp3 (Fig. 1C). However, no protein band for TGBp3 was observed in the leaves infected with PMTV in repeated experiments (data not shown), most probably due to a low concentration of the protein and inadequate sensitivity of the antibodies. Therefore, the cDNA of PMTV RNA3 was engineered (designated as PMTV21K-Myc) to express TGBp3 with a C-terminal Myc tag sequence...
(Fig. 1A) and subsequently co-inoculated with the PMTV RNA1 and RNA2 into N. benthamiana leaves. The upper non-inoculated leaves systemically infected with PMTV21K-Myc were sampled 18 dpi and extracts analyzed by western blotting using α-myc. A protein with the expected electrophoretic mobility of the TGBp3 was detected (Fig. 1D). No such signal was detected in the mock-inoculated control plant (Fig. 1D). The progeny viruses in the leaf samples infected with PMTV21K-Myc were analyzed by RT-PCR and sequencing of the products, which revealed an intact Myc tag sequence downstream of the TGBp3 gene. These data showed that TGBp3 was expressed in PMTV-infected N. benthamiana plants.

**PMTV TGBp3 is phosphorylated by plant tyrosine kinase activity**

TGBp3 was expressed in E. coli as an N-terminal GST-fusion protein and purified to near-homogeneity by affinity chromatography using glutathione-sepharose (Fig. 2A). The purified protein (GST-21K) was assayed for phosphorylation in a reconstituted system in which GST-21K was incubated in a kinase buffer with freshly prepared leaf extracts of N. benthamiana in the presence of [γ-32P]ATP. Analysis of the reaction by SDS-PAGE and autoradiography revealed a single radiolabeled band corresponding to the expected size of GST-21K (~48 kDa), whereas no such signal was detected in the control reaction to which only GST was added (Fig. 2B). Equal loading of GST-21K and GST in the samples was verified by Coomassie Blue staining (Fig. 2B, lower panel). The short exposure time resulting in detectable signals for the recombinant GST-21K added to the crude leaf extract was insufficient to reveal signals for endogenous tyrosine phosphorylated host proteins, which were detected by western blot analysis using α-pY (Fig. 2D and Fig. 3).
Subsequently, purified GST-21K was incubated with leaf extracts, as above, but in the presence of unlabeled ATP. The sample was subsequently divided into two aliquots, of which one was treated with λ protein phosphatase that removes phosphate groups from serine, threonine, and tyrosine residues. Western blot analysis of the proteins using α-pY revealed a band corresponding to GST-21K only in the untreated aliquot of the sample (Fig. 2C); no protein band was detected in the aliquot treated with λ-protein phosphatase (Fig. 2C). Equal loading of GST-21K in the samples was verified by Ponceau S staining (Fig. 2C). These results from two types of experiments each done three times provided evidence that TGBp3 of PMTV was phosphorylated on tyrosine by plant kinases in vitro. The leaf samples from PMTV_{21K-Myc}-infected and mock-inoculated plants tested for presence of TGBp3 with α-myc (Fig. 1D) were also tested with α-pY to examine whether TGBp3 was phosphorylated in vivo. For this purpose all samples were loaded in duplicate for analysis by SDS-PAGE. The membrane was cut to two halves each containing a similar set of samples. Development of one half of the membrane with α-pY (Fig. 2D) and the other half with α-myc (Fig. 1D) revealed a protein band at the same position corresponding to 21 kDa. No such protein band was detected in the leaves of non-infected control plants (Figs. 1D, 2D). This experiment was repeated four times independently with similar results. These data indicated that TGBp3 was phosphorylated on tyrosine by plant kinase activity in the PMTV-infected plants of _N. benthamiana_. Furthermore, leaves of _N. benthamiana_ were agroinfiltrated to express Myc-tagged TGBp3 (21K-myc). The extracts from the infiltrated leaf tissues were subjected to western blotting using α-pY. Results revealed a tyrosine-phosphorylated protein with an electrophoretic mobility corresponding to 21K (Fig. 3, lane 6, upper panel). No such protein band was detected
in the mock-infiltrated control leaves (Fig. 3, lane 2, upper panel). Western blot analysis of the same samples using α-myc confirmed that the protein band detected with α-pY corresponds to TGBp3 (21K) (Fig. 3, lane 6, lower panel). α-pY detected many unknown tyrosine phosphorylated host proteins (Fig. 3, upper panel), and also α-myc reacted with some host proteins with a considerably higher molecular weight than 21K-myc (Fig. 3, lower panel). The experiment was done three times independently with similar results.

**Phosphorylation sites in the TGBp3 of PMTV**

NetPhos2 and Scansite software prediction of the phosphorylation sites in PMTV TGBp3 suggested phosphorylation of the tyrosine residues at positions 89 and 120 within the YQDLN and QXXPF/Y motifs, respectively (Fig. 1B), which are conserved in the TGBp3 proteins of the hordei-like viruses (33). Therefore, in subsequent experiments the TGBp3 gene (tagged with Myc) was mutated using site-directed alanine substitutions at (i) Y$_{87-89}$ (mutant 21K$_{87-89A}$), (ii) Y$_{120}$ (mutant 21K$_{120A}$), and (iii) the combined substitutions (mutant 21K$_{87-89A/120A}$). All the three adjacent tyrosine residues 87–89 were substituted in order to exclude the possibility that loss of phosphorylation at residue 89 would be complemented by phosphorylation of Y$_{87}$ and/or Y$_{88}$.

The wt and mutated TGBp3-Myc genes introduced into *N. benthamiana* leaves by agroinfiltration showed similar levels of protein expression, as detected by western blot analysis with α-myc 3 days post-infiltration (Fig. 3, lower panel). The same samples were subjected to western blot analysis using α-pY (Fig. 3, upper panel). The wt TGBp3 (21K-myc, lane 6) and mutants 21K$_{87-89A}$ (lane 5) and 21K$_{120A}$ (lane 4) were found to be phosphorylated on tyrosine. In contrast, no tyrosine phosphorylation of TGBp3 was detected in leaves expressing 21K$_{87-89A/120A}$ (lane 3), the PMTV 8K protein (control, lane 1), or in the mock-infiltrated leaves (lanes 2), whereas tyrosine
phosphorylated host proteins were detected. These results from three independent experiments indicated that the tyrosine residue at position 120 and at least one of the tyrosine residues at the positions 87–89 were phosphorylated.

**Mutation of the phosphorylation sites in TGBp3 impairs PMTV virulence**

The tyrosine residues at positions 87–89 and 120 were substituted for alanine also in TGBp3 in the full-length cDNA of PMTV RNA3, which gives rise to PMTV infection when the *in vitro* transcripts are co-inoculated into plants with the RNA1 and RNA2 transcripts produced from their respective plasmids (34). Plants of *N. benthamiana* were mechanically co-inoculated with the PMTV RNA1 and RNA2 transcripts and those of either PMTV<sub>21K</sub> or the mutated constructs pPMTV<sub>21K</sub>87–89A, pPMTV<sub>21K</sub>120A, or pPMTV<sub>21K</sub>87–89A/120A. In three independent experiments, systemic infection was detected by RT-PCR at 7 dpi in plants inoculated with wt PMTV (Fig. 4A). In contrast, no systemic infection was detected in plants in which the mutant transcripts were used, as tested by RT-PCR (Fig. 4A) and DAS-ELISA (data not shown) at 14 and 18 dpi, respectively. RT-PCR analysis of the inoculated leaves revealed readily detectable amplification products of the expected size in leaves infected with wt PMTV, whereas no products or only very faint bands were detected in the leaves inoculated with the mutant constructs (Fig. 4A, and data not shown). These results indicated that the mutations introduced into the phosphorylation sites in TGBp3 significantly decreased infectivity of PMTV.

**Cell-to-cell movement of PMTV is impaired by mutation of the phosphorylation sites in TGBp3**
Cell-to-cell movement was studied by introducing the mutated TGBp3 genes into an engineered cDNA of PMTV RNA3 that expressed GFP as an N-terminal fusion with TGBp1 (Fig. 1A). Development of virus infection in the inoculated leaves was monitored under a hand-held UV lamp and by confocal microscopy. Lesions consisting of many epidermal cells became visible in leaves inoculated with wt GFP-PMTV at 3 dpi (Fig. 4B). In leaves inoculated with GFP-PMTV21K87–89A, individual fluorescent epidermal cells were detected, indicating that the mutant virus was infectious but debilitated in cell-to-cell movement (Fig. 4B). RT-PCR analysis of the leaves and subsequent sequencing of the products confirmed that the virus had retained the introduced mutations. These results indicated that mutation of this site of TGBp3 was detrimental to cell-to-cell movement of PMTV. In contrast, no fluorescence was observed in leaves inoculated with the mutant viruses GFP-PMTV21K120A or GFP-PMTV21K87–89A/120A at any time up to 18 dpi. The results obtained by confocal microscopy were consistently observed in a total of 12 leaves inoculated with each virus in three independent experiments.

Influence of TGBp3 phosphorylation sites on interactions with TGBp2

Previous studies with yeast two-hybrid assays have shown that PMTV TGBp3 and TGBp2 interact (24, 25), and this interaction is suggested to be needed for viral movement (15). In the present study, the wt and mutated TGBp3 genes of PMTV were transferred into the yeast two-hybrid assay vectors for expression as a fusion protein with the activation domain (AD), whereas TGBp2 was cloned in the binding domain (BD) vector. The yeast was co-transformed with the BD-TGBp2-containing plasmid and one of the AD-TGBp3-containing plasmids. After selection on selective medium (Fig. 5A), co-transformants were transferred to a new selective medium allowing the growth of only those transformants in which the selective marker genes were
activated by interaction of the pairs of proteins tested (Fig. 5B). Interactions were detected between TGBp2 and wt TGBp3 as well as between TGBp2 and the mutated TGBp3 proteins: 21K87–89A, 21K120A, and 21K87–89A/120A (Fig. 5B, lanes 1 to 4, respectively). None of the constructs activated reporter genes autonomously in yeast (Fig. 5B, lanes 5–9). Expression of the fusion proteins in yeast cells was confirmed by western blot analysis (Fig. 5E).

Quantitative differences in the strength of interactions were studied by co-transforming the plasmids in the aforementioned combinations into another yeast strain that encoded β-galactosidase under a strong promoter, facilitating the β-galactosidase measurements using a microplate assay (42). Although interactions between TGBp2 and wt TGBp3 or 21K120A (Fig. 5, lanes 1 and 3, respectively) were sufficient for growth of yeast on the selective medium (Fig. 5B), the interactions were weak, as estimated by the β-galactosidase assay (Fig. 5C, D). In contrast, the TGBp3 mutants 21K87–89A and 21K87–89A/120A (Fig. 5, lanes 2 and 4, respectively) showed an enhanced interaction with TGBp2, as suggested by a 10-fold higher β-galactosidase activity than observed with the wt TGBp3 (Fig. 5C, D).

**DISCUSSION**

Plant viruses encode MPs for intra- and intercellular transport of viral ribonucleoprotein complexes. A few viral MPs are known to be phosphorylated by serine/threonine kinases, but detailed understanding of how phosphorylation modulates their functions is limited (8, 9, 10, 11, 12, 13, 14, 50). Furthermore, it is unclear whether tyrosine kinases participate in phosphorylation of viral MPs. Viruses such as PMTV encode three movement-associated TGB proteins acting coordinately. TGBp3 plays an important role in the intracellular viral movement by targeting the
viral ribonucleoprotein complex to plasmodesmata for cell-to-cell movement (15). However, the relative abundance of TGBp3 is the lowest among the TGB proteins in infected cells, which makes it difficult to detect (27, 29, 30, 32, 51). Indeed, PMTV TGBp3 has previously been detected in leaf tissues only following over-expression from a heterologous viral vector (24). In our present study, however, PMTV TGBp3 was also detected in PMTV-infected plants. Furthermore, our study revealed that PMTV TGBp3 was phosphorylated by plant tyrosine kinase activity in infected plants.

The luminal motifs in the central part (87YYYQDLN93) and the C-proximal part (116QEFPYGN123) of PMTV TGBp3 (25) were found to be sites of tyrosine phosphorylation. Substitution of both the tyrosine residues Y87–89 and Y120 with alanine residues abolished phosphorylation of TGBp3, in contrast to the substitutions introduced at only one of the two sites, indicating that both sites were phosphorylated. These results also indicated that only these two sites were tyrosine phosphorylated in TGBp3. These mutations that abolished tyrosine phosphorylation of TGBp3 resulted in loss of any discernible PMTV infectivity in N. benthamiana. In contrast, substitution of only Y87–89 to alanine did not abolish infectivity but allowed high levels of virus multiplication in initially infected cells, as evidenced by GFP fluorescence following inoculation with GFP-PMTV21K87–89A. However, infection with GFP-PMTV21K87–89A was restricted to the initially infected cells. This result is in agreement with a previous study showing that substitution of Y89 for glycine in the TGBp3 of PMTV prevented targeting of TGBp3 to plasmodesmata, inhibited viral cell-to-cell movement, and resulted in infection of only individual cells (25). Our results also show that the defective cell-to-cell movement of PMTV was associated with alteration in the strength of the interactions between TGBp3 and TGBp2. Upon mutation of Y87–89 to alanine, the interaction between the TGBp3 and
TGBp2 of PMTV was enhanced by an order of magnitude. In a previous study, substitution of Y89 for glycine in TGBp3 did not appear to affect the interaction with TGBp2 (25), probably because the interaction was enhanced rather than inhibited and no quantitative assay was used. Taken together, the previous studies are consistent with our findings in terms of the importance of interactions between TGBp3 and TGBp2 in the movement of PMTV (24, 25) and BSMV (26, 27) and our findings now suggest that tyrosine phosphorylation may be the mechanism regulating the interaction between TGBp3 and TGBp2.

The current model of PMTV movement suggests that the putative viral movement complexes, which supposedly include TGBp2, TGBp3, and a ribonucleoprotein complex containing TGBp1 and viral RNA, associate with motile membrane compartments of the endoplasmic reticulum via the two transmembrane domains located at the N-proximal and C-terminal parts of TGBp3. The movement complex is targeted to plasmodesmata along the endoplasmic reticulum–actin network by TGBp3, and the ribonucleoprotein complex is passed through plasmodesmata to the adjacent cell. TGBp2 and TGBp3 gate plasmodesmata open but do not follow the ribonucleoprotein complex and are instead recycled via an endocytotic pathway (23, 25). The reversible nature of phosphorylation (6) may therefore provide the means for the proposed recycling and dynamic interaction between TGBp3 and TGBp2, allowing TGBp3 to participate in multiple intracellular viral transport events.

Substitution of Tyr(120) for alanine in TGBp3 resulted in an apparent loss of infectivity of PMTV in N. benthamiana. However, phosphorylation of TGBp3 was not lost by the mutation, owing to phosphorylation at the other site (Tyr57–60). Also, no discernible effect on the TGBp3-TGBp2 interaction was associated with the Tyr120Ala mutation, as tested by the yeast two-hybrid assay. The existence of two distant tyrosine phosphorylation sites in TGBp3, both
important for the infection cycle of PMTV, suggests that they may be needed to achieve threshold levels of TGBp3 phosphorylation required for different MP functions. Tyr(89) and Tyr(120) lie within putative tyrosine-based sorting motifs of TGBp3 (YXXΦ; where Φ is a bulky hydrophobic amino acid). The sorting motif is important for protein endocytosis, which in some proteins is controlled by phosphorylation of the tyrosine residue of the motif (52, 53, 54). The motif $^{89}$YQDL, located in the lumenal central part of TGBp3, is conserved among the hordei-like viruses and is implicated in the endocytosis of TGBp2 and TGBp3 (23, 25). In contrast, the lumenal $^{120}$YGNI of PMTV TGBp3 (25) is less conserved among the other hordei-like viruses. The endocytotic pathways also play a role in the functions of MPs in other plant viruses, including the 30K MP of TMV and the MP of *Cabbage leaf curl virus* (genus *Begomovirus*) (55). However, the role of phosphorylation in the regulation of endocytosis of these MPs has not been reported.

The MPs of a few (+)ssRNA viruses are phosphorylated by serine- and threonine-specific protein kinases. Phosphorylation of the C-terminal Ser(258) or Ser(265), or the Thr(261), enhances the 30K MP–mediated cell-to-cell movement of TMV, whereas phosphorylation of multiple sites reduces or inhibits TMV movement (9). In the genus *Potyvirus*, coat protein is one of the proteins facilitating viral cell-to-cell and long-distance movement in plants. The CKII kinase of tobacco phosphorylates Thr$^{242}$ in the coat protein of PVA, which inhibits viral movement (13). TGBp1 of *Potato virus X* is phosphorylated on Ser$^{165}$ in tobacco, possibly by CKII, but the functional significance of phosphorylation is unknown (16). The results of our study expand the comprehension of phosphorylation-mediated regulation of viral MPs significantly by implicating also tyrosine phosphorylation in the process.
Our study links tyrosine kinases to virus infection in plants by showing that one of the TGB proteins, TGBp3, undergoes tyrosine phosphorylation, which is novel for TGB-containing viruses and plant virus proteins in general. This fundamental novel finding opens a new, unexploited arena to study interaction of viruses and plants and understand how viruses utilize host factors to advance infection. Tyrosine phosphorylation was previously considered to be rare in plants, but recent studies indicate otherwise. Up to 4.3% of all phosphorylation events of proteins in *Arabidopsis thaliana* may occur on tyrosine, which is a comparable frequency to tyrosine phosphorylation in animals (56, 57). Furthermore, over 75% of the tyrosine-phosphorylated proteins in *Arabidopsis* are phosphorylated at multiple sites (56). Considering how viruses depend on and utilize cellular factors during the infection cycle, tyrosine phosphorylation of MPs may be more common than previously thought. Proteins of mammalian viruses including vaccinia, variola and monkeypox are phosphorylated by tyrosine kinases, and kinase inhibitors can increase survival of the host by reducing viral load and dissemination of the virus to distal tissues (58, 59). It is conceivable that inhibition of tyrosine kinases required for phosphorylation of viral proteins could have a role to play in combating viral infections also in plants.

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REFERENCES


FIGURE LEGENDS

FIG. 1. Schematic representation of PMTV RNA3 and detection of PMTV TGBp3 in leaf tissues of *N. benthamiana* by western blotting. (A) PMTV RNA3 with positions of the open reading frames (ORFs) for the triple gene block proteins (TGBp1 - 51K; TGBp2 - 13K; and TGBp3 - 21K) and the 8-kDa cysteine-rich protein (8K) depicted. Insertion sites for the green fluorescent protein (GFP) gene, expressed with two additional amino acids (GFP-Gly-Asn) linking GFP to TGBp1, and for the Myc epitope sequence expressed with a three–amino acid spacer (Glu-Phe-Gly-Myc) are shown. (B) Schematic representation of PMTV TGBp3 showing the amino acid motifs containing the tyrosine residues (87–89 and 120) mutated for alanine in this study. The amino acid residues conserved among the hordei-like viruses (bold), and the putative tyrosine-based YXXΦ sorting motifs (underlined) are shown. Dashed lines indicate the end of the TGBp2 ORF and the beginning of the 8K ORF, which overlap with the TGBp3 ORF. (C) Leaves agroinfiltrated with a 35S promoter–driven plant expression vector for TGBp3 over-expression (21K) and tested 3 days post-infiltration using the polyclonal antibodies (α-21K) raised against PMTV TGBp3. Mock leaves were infiltrated using infiltration buffer alone. (D) Leaves systemically infected with PMTV_{21K-Myc}, in which TGBp3 was expressed as a fusion with a Myc tag, and detected using anti-Myc monoclonal antibody (α-myc) at 18 dpi. Mock-inoculation was done with buffer only. Position of 21K (arrowhead) is indicated in C and D.

FIG. 2. *In vitro* and *in vivo* phosphorylation of PMTV TGBp3. (A) GST-tagged PMTV TGBp3 (GST-21K) (arrowhead) expressed in *E. coli*, purified and analyzed by SDS-PAGE. The gel was stained with Coomassie Blue. The molecular mass markers (M) and their sizes (in kilodaltons;
left side) are indicated. (B) In vitro kinase assay of the GST-21K fusion protein and GST alone (control) by incubation in an *N. benthamiana* leaf extract diluted in kinase buffer in the presence of [γ-33P]ATP. Samples were separated by SDS-PAGE (12.5% w/v acrylamide), stained with Coomassie Blue (lower panel) and analyzed by autoradiography (upper panel). The positions of molecular mass markers (in kilodaltons; left side) are indicated. Filled and open arrowheads indicate the positions of GST-21K and GST, respectively. (C) Phosphorylation of GST-21K by plant protein kinase activity. GST-21K (0.5 μg) was incubated with leaf extracts from *N. benthamiana* in the presence of unlabeled ATP. Subsequently, half of the sample was treated with λ-protein phosphatase (+λ PPase), and half was left untreated (−λ PPase). Western blotting was done using anti-phosphotyrosine MAb (α-pY). Equal loading of proteins was verified by Ponceau S staining. The position of the 50-kDa molecular marker is indicated. (D) Tyrosine-phosphorylation of PMTV TGBp3 in leaves of *N. benthamiana* infected with PMTV21K-Myc as detected by western blot analysis using α-pY (tyrosine phosphorylated, higher molecular weight host proteins were also detected by α-pY). Fig. 1D shows detection of PMTV21K-Myc in the same sample using α-myc. The positions of molecular mass markers (in kilodaltons; right side) and position of 21K (arrowhead) are indicated.

FIG. 3. Tyrosine-phosphorylation of PMTV TGBp3 expressed in leaves of *N. benthamiana* by agroninfiltration. Detection of myc-tagged PMTV TGBp3 (21K-myc) and myc-tagged 21K mutants was carried out by western blot analysis using α-pY (upper panel) and α-myc (lower panel). Positions of molecular mass markers are shown at the right. Position of 21K-myc is indicated with an arrowhead. Lane 1, leaf tissue agroinfiltrated for expression of the PMTV 8K protein (8K), and lane 2, leaf tissue mock-infiltrated with infiltration buffer alone are negative.
controls, whereas lane 6, leaf tissue infiltrated for expression of 21K-myc is a positive control. 

Lane 3, TGBp3 with tyrosine residues at positions 87–89 and 120 substituted for Ala (21K87–89A/120A); lane 4, TGBp3 with the tyrosine residue at position 120 substituted for alanine (21K120A); and lane 5, TGBp3 with tyrosine residues at positions 87–89 substituted for alanine (21K87–89A). α-pY and α-myc detected also host proteins with molecular weight higher than 21K-myc.

FIG. 4. Effect of amino acid substitutions in TGBp3 on the infectivity of PMTV mutants. (A) Detection of PMTV infection in the inoculated leaves (I) and upper non-inoculated leaves (S) of N. benthamiana. Samples were analyzed by RT-PCR using primers specific to the TGBp3 sequence at 3, 7, 10, and 14 dpi. M, DNA molecular size markers, with the 1000 bp and 500 bp markers indicated. (B) Confocal microscopy of N. benthamiana leaves inoculated with PMTV that express GFP fused to TGBp1. GFP-PMTV expresses wt TGBp3, whereas GFP-PMTV21K87–89A expresses TGBp3 where tyrosine residues at position 87–89 were replaced with alanine. Images were captured at 3 dpi. Bars, 100 µm.

FIG. 5. Yeast two-hybrid analysis of the interactions between TGBp2 (13K) and the mutated forms of TGBp3 (21K) of PMTV. (A) Co-transformation of the binding domain (BD) and activation domain (AD) vector plasmids is indicated as growth of the yeast on selective medium. The plasmids were selected by the lack of tryptophan (-Trp) and leucine (-Leu), respectively, in the culture medium. TGBp2 was expressed from the BD plasmid, and the TGBp3-based constructs were expressed from the AD plasmid, respectively, in the combinations indicated above the panel. (ni), no insert; + and -, the positive and negative controls of the Clontech yeast two-hybrid system. (B) Growth of the co-transformed yeast strains on the selective medium.
indicating interactions between the co-expressed PMTV proteins. The culture medium lacked adenine (-Ade), histidine (-His), leucine (-Leu) and tryptophan (-Trp). (C) An X-gal assay in a 96-well microtiter plate format (42) was used to estimate the strength of the protein-protein interactions, as detected by intensity of the blue color resulting from the X-gal substrate processed by β-galactosidase activity in co-transformed yeast cells. Enhanced interaction between the test proteins resulted in enhanced expression of β-galactosidase and of blue color. (D) Quantification of the intensity of the blue color in the yeast cells observed in (C) using ImageJ software. Columns indicate β-galactosidase activity relative to the positive control (+) of the yeast two-hybrid system provided by Clontech. Error bars indicate the standard deviation of the mean (n = 3). (E) Expression of the 13K-BD and 21K-AD fusion proteins in yeast cells, as tested by western blot analysis using MAbs specific to the BD and AD domains, respectively. Ponceau S staining of the membrane was used as a protein loading control. Positions of molecular mass markers (in kilodaltons; right side) are indicated.