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 the 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 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.
proteins present in infected plant tissues are extremely difficult to detect due to a lower titer (26, 29, 31, 32), and previous attempts to detect and localize 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.

TGBp3 in hordei-like viruses contains a conserved YQDLN motif in the central part of the protein (33). The YQDLN motif in PMTV TGBp3 serves a critical role during infection of plants. When the motif is mutated to GQDGGN, 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. 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 coinoculated with the RNA1 and RNA2 transcripts into plants (34).

The putative phosphorysine sites in PMTV TGBp3 were predicted by using NetPhos 2 and ScanSite. The NetPhos 2 algorithm is a neural network model with a false-positive prediction rate of 0 to 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 previously (37). Tyr-to-alanine (Ala) substitutions were introduced into the residues Tyr87, Tyr88, and Tyr120 (construct pPMTV3 21K87-89A or TGBp3 21K120A, pPMTV3 21K120A) (Fig. 1A and B). Furthermore, all mutations were combined in the construct pPMTV3 21K87-89A/120A (Fig. 1B). The primers used to prepare the above-mentioned constructs and other constructs in this study are available upon request.

The sequence encoding the Myc epitope (EQKLISEEDL) was added to the 3′ end and TGBp3 (pPMTV3 21K-Myc) (Fig. 1A). To produce green fluorescent protein (GFP) fusion constructs of pPMTV3, an NcoI site was created at the 5′ end of the TGBp3, a GFP coding sequence was amplified from PVA-GFP (38) and subsequently inserted at the 5′ end of the 21K protein gene of wild-type (wt) or mutant pPMTV3 constructs (Fig. 1A) to obtain GFP-pPMTV3, GFP-pPMTV3 21K87-89A, GFP-pPMTV3 21K120A, and GFP-pPMTV3 21K87-89A/120A.

To express PMTV TGBp3 in fusion with glutathione S-transferase (GST) (GST 21K), the 21K protein gene was cloned into the pGE6P-1 expression vector (Amersham Biosciences, Piscataway, NJ) into construct 21KpGEX6P-1. For expression in plants under the Cauliflower mosaic virus 35S promoter, the 21K-Myc and 8K genes of PMTV were cloned into the binary vector pH1 (39). Cloning was done according to standard molecular biology protocols (40). Construct 21KpMyc was used as the parental plasmid for site-directed mutagenesis to obtain the mutated genes for expression of 21K 87-89A, 21K 120A, and 21K 87-89A/120A proteins as Myc-tagged proteins from binary vectors in plants.

For yeast two-hybrid system (YTHS) analysis, the mutated TGBp3 gene sequences encoding the 21K 87-89A, 21K 120A, and 21K 87-89A/120A proteins were obtained by PCR from parental plasmids pPMTV3 21K 87-89A, pPMTV3 21K 120A, and pPMTV3 21K 87-89A/120A respectively, using Dynazyme II DNA polymerase (Finnzymes) according to manufacturer’s instructions. The genes for the 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), and plasmid DNA was then prepared and cleaved with appropriate restriction enzymes for subsequent cloning into the similarly cleaved pGADT7 and pGBK7 vectors (Clontech, Mountain View, CA). 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 at 600 nm (OD600) of 0.6 in Luria broth (Sigma-Aldrich, St. Louis, MO). Protein expression was induced by addition of isopropyl-β-D-thiogalactopyranoside (Promega) to a final concentration of 0.1 mM. The cultures were grown for an additional 3 h (OD600 = 1.5). The cells were harvested by centrifugation at 6,000 × g for 10 min and resuspended in PBS containing 1 mM benzamidine and 1 mM phenylmethylsulfonyl fluoride (Sigma-Aldrich).

The cells were lysed by sonication on ice. The soluble proteins were precipitated with 15% (wt/vol) trichloroacetic acid (TCA) at 4°C for 1 h. After centrifugation at 12,000 × g for 15 min, the supernatants were dialyzed against 0.1 M Tris-Cl, pH 8.0, and the samples were further purified using polyethylene glycol 20K (PEG 20K) precipitation (41). The purified samples were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and the desired bands were excised and identified by mass spectrometry (42) and/or Western blotting. The presence of the epitope in the recombinant proteins was confirmed by Western blotting using anti-Myc antibodies (Sigma-Aldrich).
mM, and the cells were grown for 3 h at 30°C and collected by centrifugation (5,000 × 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 the 21K protein 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) according to the manufacturer’s instructions.

**In vitro and in vivo kinase assays and Western blotting.** Leaf tissue from full-grown leaves of 5-week-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 10 μM kinase buffer were mixed in a 10:1 ratio (final concentration of 20 mM HEPES-KOH [pH 7.4], 10 mM MgCl₂, 50 mM NaCl, 1 mM dithiothreitol, and 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 [γ-<sup>32</sup>P]ATP (Amersham Biosciences) for 45 min at 37°C. The 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% [wt/vol] acrylamide) and subjected to autoradiography.

In another experiment, 500 ng of purified GST-21K was incubated as described 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) according to the manufacturer’s protocols. The samples were subjected to SDS-PAGE (12.5% [wt/vol] 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 at 3 days postinfiltration, ground in SDS-PAGE sample buffer at a 1:3 ratio (wt/vol) at room temperature, and heated to 95°C for 5 min. Aliquots of 20 μl were analyzed by SDS-PAGE (12.5% [wt/vol] acrylamide), after which proteins were transferred onto a polyvinylidene fluoride membrane (Amersham Biosciences). Western blotting was done by using a specific anti-phosphotyrosine (anti-pY) (clone 4G-10 Platinum; Upstate, Millipore) or anti-Myc (clone 9E10; Santa Cruz Biotechnology, Santa Cruz, CA) monoclonal antibody (MAb) or polyclonal antibodies to the PMTV 21K protein (prepared in this study). Polyclonal rabbit anti-mouse immunoglobulins (IgG) conjugated with horseradish peroxidase (HRP) (Dako Denmark A/S, Glostrup, Denmark) and HRP-conjugated anti-rabbit donkey IgG (GE Healthcare, Little Chalfont, United Kingdom) were used as secondary antibodies. Each membrane was developed with the SuperSignal West Femto chemiluminescence detection system (Thermo Scientific, Rockford, IL).

**YTHS assay.** Competent yeast cells were prepared and transformed by using Matchmaker GAL4 Two-Hybrid System 3 (Clontech) with *Saccharomyces cerevisiae* strain AH109 and a small transformation scale according to the manufacturer’s instructions. Cotransformations were made with different combinations of pGADT7 activation domain (AD) and pGBK7 DNA-binding domain (BD) constructs. Cotransformation of pGBK7-53 (Clontech) with pGADT7-T (Clontech) was used as a positive interaction control, and cotransformation of pGBK7-Lam (Clontech) with pGADT7-T was used as a negative interaction control. To select for cotransformants, the cells were plated on supplemental dropout medium lacking leucine and tryptophan (SD/–Leu/–Trp) and grown at 30°C for approximately 36 h. The colonies were then replated onto supplemental dropout medium lacking adenine, histidine, leucine, and tryptophan (SD/–Ade/–His/–Leu/–Trp) to check for interactions between the two-hybrid proteins, as indicated by growth on the selection plate after a 1-week incubation at 30°C.

To test the strength of the interactions, yeast strain Y187 was cotransformed with the above-described constructs, and the pellet 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) β-galactosidase (β-gal) assay was performed and analyzed as described previously (42). The intensity of the color, caused by cleavage of X-gal by β-gal activity, was determined with ImageJ software (43). These intensities were used to calculate β-gal activity relative to that of the positive control, which 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 previously (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 isolations, 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% (wt/vol) 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 by 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 MgCl₂, 10 mM morpholineethanesulfonic acid [MES], 200 μM acetylserine) at a final OD<sub>600</sub> 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 pMD RNA constructs as described previously (34). Linearized cDNA (2 μg) was used in each transcription reaction mixture. 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<sub>HPO</sub> (pH 9.2), 0.25% (wt/vol) bentonite (Sigma-Aldrich), and 0.25% (wt/vol) 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 GKP buffer.

Inoculated and upper leaves were collected at 18 days postinoculation (dpi). Samples were tested for the presence of PMTV by a double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA), using a MAB specific to the PMTV coat protein (Science and Advice for Scottish Agriculture [SASA], Edinburgh, United Kingdom), as described previously (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 by using a TRizol-like reagent (48). RNA samples were treated with RNase-free DNase (Promega) at 37°C for 30 min. cDNA was synthesized with Moleoney 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 by using Phusion DNA polymerase (Finnzymes) according to the manufacturer’s instructions.

In another experiment, plants were coinoculated with wt RNA1 and RNA2 and wt or mutant GFP-RNA3. Infections were monitored daily up to 18 dpi by using a handheld 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 previously (49). Each single bombardment contained 0.1 mg gold particles (1-μm diameter; Bio-Rad, Hercules, CA) 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 by using coverslips and tape and mounted in water. Pictures were taken with a Leica TCS SP5II HGS A confocal microscope using an HC PL Apo 10×/0.4 objective. GFP was visualized by using argon laser excitation at 488 nm and an acquisition window of 500 to 552 nm. The LAS AF Lite software package (Leica Mi-
RESULTS

Detection of TGBp3 in PMTV-infected plant tissues. Extracts from *N. benthamiana* leaves infected with PMTV or agroinfiltrated for 35 S 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 overexpressing 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 PMTV21K-Myc) to express TGBp3 with a C-terminal Myc tag sequence (Fig. 1A) and subsequently coinoculated with PMTV RNA1 and RNA2 into *N. benthamiana* leaves. The upper noninoculated leaves systemically infected with PMTV21K-Myc were sampled at 18 dpi, and extracts were analyzed by Western blotting using anti-Myc antibody (α-Myc). A protein with the expected electrophoretic mobility of 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 mixture, 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, bottom). The short exposure time resulting in detectable signals for the recombinant GST-21K protein 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 anti-pY antibody (α-pY) (Fig. 2D and 3).

Subsequently, purified GST-21K was incubated with leaf extracts, as described above, but in the presence of unlabeled ATP. The sample was subsequently divided into two aliquots, one of which 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 PMTV21K-Myc-infected and mock-inoculated plants tested for the 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 into 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 noninfected control plants (Fig. 1D and D). This experiment was repeated four times independently, with similar results. These data indicated that TGBp3 was phos-
Tyrosine phosphorylation of PMTV TGBp3 expressed in leaves of *N. benthamiana* by agroinfiltration. Detection of Myc-tagged PMTV TGBp3 (21K-myc) and Myc-tagged 21K mutants was carried out by Western blot analysis using anti-pY (top) and anti-Myc (bottom). Positions of molecular mass markers are shown at the right. The position of 21K-Myc is indicated with an arrowhead. Lanes 1 and 2, leaf tissue agroinfiltrated for expression of the PMTV 8K protein (8K) (lane 1) and leaf tissue mock infiltrated with infiltration buffer alone (lane 2) as negative controls; lane 6, leaf tissue infiltrated with *N. benthamiana* leaves inoculated with wt GFP (control) (lane 1), or the mock-infiltrated leaves (lane 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 positions 87 to 89 were phosphorylated by in vitro transcription 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 the 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, which gives rise to PMTV infection when the *in vitro* transcripts are coinoculated into plants with the RNA1 and RNA2 transcripts produced from their respective plasmids (34). Plants of *N. benthamiana* were mechanically coinoculated with the PMTV RNA1 and RNA2 transcripts and those of either PMTV-21K or the mutated construct pPMTV21K87-89A, pPMTV21K120A, or pPMTV21K87-89A/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 the infectivity of PMTV.

**Mutation of the phosphorylation sites in TGBp3 impairs PMTV virulence.** The tyrosine residues at positions 87 to 89 and 120 were also substituted for alanine in *TGBp3* in the full-length cDNA of PMTV RNA3, which gives rise to PMTV infection when the *in vitro* transcripts are coinoculated into plants with the RNA1 and RNA2 transcripts produced from their respective plasmids (34). Plants of *N. benthamiana* were mechanically coinoculated with both the RNA1 and RNA2 transcripts and those of either PMTV-21K or the mutated construct pPMTV21K87-89A, pPMTV21K120A, or pPMTV21K87-89A/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 the infectivity of PMTV.

**Phosphorylation sites in TGBp3 of PMTV.** NetPhos 2 and ScanSite software prediction of the phosphorylation sites in PMTV TGBp3 suggested phosphorylation of the tyrosine residues at positions 87 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 by using site-directed alanine substitutions at (i) Y$_{87-89}$ (21K$_{87-89A}$ mutant), (ii) Y$_{120}$ (21K$_{120A}$ mutant), and (iii) the combined substitutions (21K$_{87-89A/120A}$ mutant). All three adjacent tyrosine residues 87 to 89 were replaced 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 at 3 days postinfiltration (Fig. 3, bottom). The same samples were subjected to Western blot analysis using α-pY (Fig. 3, top). wt TGBp3 (21K-Myc) (Fig. 3, top, lane 6) and the 21K$_{87-89A}$ (lane 5) and 21K$_{120A}$ (lane 4) mutants were found to be phosphorylated on tyrosine. In contrast, no tyrosine phosphorylation of TGBp3 was detected in leaves expressing the 21K$_{87-89A/120A}$ mutant (Fig. 3, top, lane 3), the PMTV 8K protein (control) (lane 1), or the mock-infiltrated leaves (lane 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 positions 87 to 89 were phosphorylated.
sults 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 GFP-PMTV21K120A or GFP-PMTV21K87-89A/120A mutant virus at any time up to 18 dpi. The results obtained by confocal microscopy were consistently observed for 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 was 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 expressed from the BD plasmid, and the TGBp3-based constructs were expressed from the AD plasmid in the combinations indicated above the panel. ni, no insert; + and −, positive and negative controls of the Clontech yeast two-hybrid system. (B) Growth of the cotransformed yeast strains on selective medium indicating interactions between the coexpressed 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 the intensity of the color resulting from the X-gal substrate processed by β-galactosidase activity in cotransformed yeast cells. Enhanced interaction between the test proteins resulted in enhanced expression of β-galactosidase and of color. (D) Quantification of the intensity of the blue color in the yeast cells observed in panel C by using ImageJ software. Columns indicate β-galactosidase activity relative to that of 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 (at right, in kilodaltons) are indicated.

Quantitative differences in the strength of interactions were studied by cotransforming the plasmids in the above-mentioned combinations into another yeast strain that encoded β-galactosidase under a strong promoter, facilitating β-galactosidase mea-
measurements using a microplate assay (42). Although interactions between TGBp2 and wt TGBp3 or the 21K120A protein (Fig. 5, lanes 1 and 3, respectively) were sufficient for growth of yeast on selective medium (Fig. 5B), the interactions were weak, as estimated by the β-galactosidase assay (Fig. 5C and D). In contrast, the 21K87-89A and 21K87-89A/120A TGBp3 mutants (Fig. 5, lanes 2 and 4, respectively) showed an enhanced interaction with TGBp2, as suggested by a 10-fold-higher β-galactosidase activity than that observed with wt TGBp3 (Fig. 5C and 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 a detailed understanding of how phosphorylation modulates their functions is limited (8–14, 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. TGB3 plays an important role in intracellular viral movement by targeting the viral ribonucleoprotein complex to plasmodesmata for cell-to-cell movement (15). However, the relative abundance of TGB3 is the lowest among the TGB proteins in infected cells, which makes it difficult to detect (27, 29, 30, 32, 51). Indeed, PMTV TGB3 has previously been detected in infected leaf tissues only following overexpression from a heterologous vector (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 TGB3. 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 endocytic 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 Tyr120 for alanine in TGB3 resulted in an apparent loss of infectivity of PMTV in *N. benthamiana*. However, phosphorylation of TGB3 was not lost by the mutation, owing to phosphorylation at the other site (Tyr87–89). 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 TGB3, both important for the infection cycle of PMTV, suggests that they may be needed to achieve threshold levels of TGB3 phosphorylation required for different MP functions. Tyr89 and Tyr120, lie within putative tyrosine-based sorting motifs of TGB3 (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 (52–54). The motif YQDL, located in the luminal central part of TGB3, is conserved among the hordei-like viruses and is implicated in the endocytosis of TGBp2 and TGBp3 (23, 25). In contrast, the luminal motif 120YGNI of PMTV TGB3 (25) is less conserved among the other hordei-like viruses. The endocytic pathways also play a role in the functions of MPs in other plant viruses, including the 30K MP of TMV and the MP of *Cabbagge 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 positive-sense ssRNA viruses are phosphorylated by serine- and threonine-specific protein kinases. Phosphorylation of the C-terminal Ser258 or Ser265 or of Thr261 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 Thr242 in the coat protein of PVA, which inhibits viral movement (13). TGBp1 of *Potato virus X* is phosphorylated on Ser165 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 also implicating 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 interactions 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 frequency comparable to that of 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 viruses, 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 also have a role in combating viral infections in plants.

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