Phosphorylation and/or Presence of Serine 37 in the Movement Protein of Tomato Mosaic Tobamovirus Is Essential for Intracellular Localization and Stability In Vivo

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The P30 movement protein (MP) of tomato mosaic tobamovirus (ToMV) is synthesized in the early stages of infection and is phosphorylated in vivo. Here, we determined that serine 37 and serine 238 in the ToMV MP are sites of phosphorylation. MP mutants in which serine was replaced by alanine at positions 37 and 238 (LQ37A238A) or at position 37 only (LQ37A) were not phosphorylated, and mutant viruses did not infect tobacco or tomato plants. By contrast, mutation of serine 238 to alanine did not affect the infectivity of the virus (LQ238A). To investigate the subcellular localization of mutant MPs, we constructed viruses that expressed each mutant MP fused with the green fluorescent protein (GFP) of *Aequorea victoria*. Wild-type and mutant LQ238A MP fusion proteins showed distinct temporally regulated patterns of MP-GFP localization in protoplasts and formation of fluorescent ring-shaped infection sites on *Nicotiana benthamiana*. However, mutant virus LQ37A MP-GFP did not show a distinct pattern of localization or formation of fluorescent rings. Pulse-chase experiments revealed that MP produced by mutant virus LQ37A was less stable than wild-type and LQ238A MPs. MP which contained threonine at position 37 was phosphorylated, but the stability of the MP in vivo was very low. These studies suggest that the presence of serine at position 37 or phosphorylation of serine 37 is essential for intracellular localization and stability of the MP, which is necessary for the protein to function.

Following replication in initially infected cells, plant viruses move to adjacent cells through plasmodesmata (6, 11, 26, 31, 38). Systemic infection takes place via the vascular tissues, from which virus spreads into other tissues (6, 10, 31). It was shown through molecular recombination experiments that the 30-kDa protein, or movement protein (MP), of tobamoviruses is involved in cell-to-cell spread of infection (32); this was confirmed by demonstrating the ability of transgenic plants expressing the MP gene [MP(+) plants] (12) to complement a viral progeny. Without the MP function, plant viruses cannot move either from cell to cell or over long distances (6, 11, 26, 31, 32).

The multiple functions or activities of MP were determined by using different several experimental approaches. By dye-coupling studies, it was demonstrated that the size exclusion limit of plasmodesmata in MP(+) plants was increased by about 10-fold compared with control plants (48). The extent of modification of the plasmodesmal size exclusion limit depended upon the MP and the host (3, 25, 47). Citovsky et al. showed that the TMV MP binds to single-stranded nucleic acids in vitro (7) and forms an elongated structure 1.5 to 2.0 nm in diameter (9). The sizes of the RNA-MP complexes are similar to the sizes of molecules that can pass through the modified plasmodesmata (estimated at 2.4 to 3.1 nm in Stokes radius). These observations together led to the proposal that tobamovirus genomic RNA is complexed with MP and passes through plasmodesmata which are enlarged by a second activity of MP, resulting in movement of genomic RNA to adjacent cells.

Tomenius et al. found by immunogold localization studies that MP accumulates in plasmodesmata of TMV-infected tobacco leaves (40). Similarly, in MP(+) transgenic plants, MP is localized to plasmodesmata (1, 15, 33). When Deom et al. analyzed subcellular fractions of leaves in MP(+) transgenic plants, they found that MP was most abundant in the cell wall fraction of older leaves whereas it was present predominantly in a crude membrane-organelle fraction and a soluble fraction in younger leaves (13). However, localization alone did not explain how the protein facilitates the cell-to-cell spread of viral progeny.

Heinlein et al. (18) established a cloned cDNA of a tobamovirus (Ob) in which the MP gene was translationally fused to the green fluorescent protein (GFP) of *Aequorea victoria*. They reported that MP is associated with microtubules in protoplasts when the MP-GFP was expressed by TMV infection. McLean et al. (27) observed a similar localization when MP-GFP was expressed under the direction of a constitutive promoter. Recently, Padgett et al. (36) described infection sites that were visualized as a fluorescent ring and reported that intracellular distribution of MP varied with the radial position of the cell within the fluorescent ring. Assuming that the outer edge of the fluorescent ring reflects an early stage of infection and that cells closer to the origin display later stages of infection, the data suggest that there are time-dependent changes of...
MP localization. Fluorescent punctate structures were present in or near the cell wall at early stages of infection, and fluorescent filaments were associated with microtubules at later stages. Heinlein et al. reported that in infected protoplasts MP-GFP was localized to the endoplasmic reticulum (ER; especially the cortical ER) and in some cases was colocalized with replecipe (19). Microtubules appear to distribute MP-GFP from the cortical ER during late stages of infection (19). MP-GFP was also observed in the cell walls in planta, and the authors proposed that association descripts the location of the MP en route to or from plasmodesmata (36). Chen et al. reported that MP may contain domains that may function independently; the region of TMV MP around amino acids 9 to 11 may be involved in targeting to ER and to plasmodesmata, the region around residues 49 to 51 may confor coalignement of the protein with microtubules, and the region around residues 88 to 101 appears to play a role in targeting to both ER and microtubules (22).

We previously demonstrated that tomato mosaic tobamovirus (ToMV) MP is phosphorylated in infected protoplasts (44). When a series of truncated MP mutants was used in a similar assay, deletion of the last 31 amino acids eliminated phosphorylation of the MP whereas deletion of the last 3 amino acids did not (44). This result suggested that the C terminus, which includes aminic residues at position 238, 257, and 261, included one or more sites of phosphorylation (44). Similar results were obtained by Citovsky et al. (8). We wanted to determine the precise location of amino acid residues that are posttranslational phosphorylation and to describe precisely the effects of phosphorylation on MP function. However, only very small amounts of MP are synthesized transiently during virus infection (42), and the amounts of phosphorylated peptides are too small to permit direct peptide sequencing. Therefore, we initiated studies to identify the amino acid residue(s) of ToMV MP that is phosphorylated in vivo by determining the incorporation of [32P] in tryptic peptides and searching for candidate amino acids in such peptides. For this purpose, we used several known ToMV MP mutants and a series of mutant MPs created by site-directed mutagenesis to localize the phosphorylated amino acid residue(s). We also examined the significance of phosphorylation by determining whether viruses that express mutant MPs are infectious, monitoring the stability of mutant MPs in vivo, and observing the intracellular localization of wild-type and mutant MP with the aid of the GFP.

MATERIALS AND METHODS

Plant materials and virus strains. Nicotiana tabacum cv. Xanthi-nc was used as a systemic host, and N. tabacum cv. Xanthi-nl was used as a local-leision host. Transgenic MP (+) plants that accumulate the TMV MP were Nicotiana tabacum cv. Xanthi-nl line 2005 (14) and N. benthamiana line H4b-3. ToMV (formerly referred to as TMV-L) was used throughout this work (34). Mutants of ToMV, AACCATGCATACAGCCACACCACCAGC, ATTCCACTCGCCGGCCACAACAAGACCGAC, and Q89SA (CTCTTGTCATAATCTTGTCGACCTTTGCAACCATAACACTC), Q75SA (CTGGTAATAATCTTGTCGACCTTTGCAACCATAACACTC), and Q18SA (CATCGACGGGGAATAATCTTGTCGACCTTTGCAACCATAACACTC) were used. ToMV mutants were constructed as described previously (23) with single-stranded DNA (ssDNA) of ER and microtubules (22).

ToMV RNA was isolated by high-yield PCR (2) with L392EcoRI and Q37AgT primers and ToPLW3 as a template. Other truncated MP was synthesized by high-yield PCR with LP37Sal and LMP3-Bam primers and ToPLW3 as a template. The PCR products were restricted with ArrIII (nt 3759) and SalI or SalI and NolI (nt 5462), respectively. The ArrIII-SalI fragment (nt 3759-5462) was replaced with a pLQ37AgT-Sal fragment (Fig. 1A). Likewise, pLQ37D, pLQ37E, and pLQ37T was established with antisense primers Q37D (AATCTTGTCGACCTTAGTAACCATAACACTC), Q37E (AATCTTGTCGACCTTTAATCAACCATAACACTC), and Q37T (AATCTTGTCGACCTTTAATCAACCATAACACTC), respectively (Fig. 1B). pLQ37TSA238 was constructed by exchanging the Ncol (nt 5462 in the genomic sequence) for fragment of pLQ37Sal and pLQ37D-Bam primers, and that of pLQ37E and pLQ37T for fragment of pLQ37Sal and pLQ37D-Bam primers.

Protoplast isolation and inoculation of viral RNAs and transcripts. Transcription reactions were performed essentially as described previously (20). Mutant transcripts and viruses are referred to by eliminating “p” from the designation of each amino acid substitution. Restriction of cDNA from wild-type MP with the aid of the GFP.

To substitute the T7 promoter for the Pum promoter of those plasmids, we used Transcription reactions were performed essentially as described previously (20). Mutant transcripts and viruses are referred to by eliminating “p” from the designation of each amino acid substitution. Restriction of cDNA from wild-type MP with the aid of the GFP.

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2-D analysis of phosphopeptides derived from MPs. Following phosphorylation, \(^{32}P\)-labeled peptides were subjected to TLC-electrophoresis in the first dimension in pH 1.9 buffer (4) for 30 min at 1.0 kV. After being air dried, the TLC plate was subjected to chromatography at room temperature for 3 h in \(-\)butanol-pyridine-glacial acetic acid-H\(_2\)O (785:607:122:486) (4).

\(\text{35S pulse-labeling of MP and pulse-chase experiments.}\\
\text{Wild-type and mutant transcripts were inoculated into BY-2 protoplasts that were subsequently cultured in the presence of 30 m\(g\) of actinomycin D per ml for 8 h; \([35S]\)methionine-[35S]cysteine protein-labeling mixture (NEN) was added to the culture medium at 1 MBq/ml for 10 min. After adding unlabeled methionine and cysteine to the culture medium, to bring the concentration of each to 1 mM, the protoplasts were rinsed and cultured in culture medium containing 1 mM each methionine and cysteine to further reduce the incorporation of radioactivity. The protoplasts were harvested at 0, 1, 3, 10, 16, 22, and 28 h after labeling and were collected. Proteins were extracted from protoplasts and subjected to SDS-PAGE (10% polyacrylamide). \(^{35}S\)-labeled MP bands were detected and traced quantitatively by a Fuji Image Analyzer.

\(\text{Construction of virus mutants containing MP-GFP fusion proteins and observation by fluorescence microscopy.}\\
\text{Before making MP-GFP fusion constructs, an S65T mutation was introduced into GFP sequences in the pObDC-GFP construct (18) to obtain increased fluorescence (17); the construct is referred to as pObDC-GFP S65T.}

\(\text{Four primers were prepared to construct MP-GFP fusions (Fig. 1A and B).}\\
\text{Primer pglyGFP (Fig. 1A) includes a SacI site (lowercase), sequences directing the synthesis of polyglycine (underlined), and N-terminal sequences from GFP:}\\
\text{AGTCAAgagctcATTTCTGGTGGTGGTATGAGTAAAGGAGAAGAA}\\
\text{CT. Primer G213 (Fig. 1A) includes a BstEII site (lowercase) and antisense GFP C-terminal sequences:}\\
\text{AAGggttaccTTATTTGTATAGTTCATCCATGCCATG.}

\text{PCRs of GFPS65T sequences were performed with the above two primers.}
and pOhA-C-GFP65T plasmid DNA as a template (2). The products were restricted with SacI and BstEII overnight. Primer L392EcoRV was previously described. Primer L2-SacI (Fig. 1B) has the sequence TACATgagCTACGCCGACCGACGTCG, which has an antisense sequence of ToMV RNA and is designed to introduce a SacI site (lowercase) following amino acid 260 of the MP and to fuse MP and GFP in the same reading frame. Each truncated MP and upstream genomic sequence was made by high-fidelity PCR (2) with L392EcoRV and L2-SacI primers, with pTLW3, pTLQ238A, pTLQ37A, pTLQ37T, or pTLQ37E as a template. The PCR products were restricted with SacI and Accl (nt 3759). The Accl (nt 3759)-BstEII (nt 5799) fragment in pTLW3 was replaced with Accl-SacI-restricted PCR fragments containing the respective region from the MP gene and a SacI-BstEII-restricted GFP fragment. The resultant viruses, LQwt-Gfus, LQ238A-Gfus, LQ37A-Gfus, LQ37T-Gfus, and LQ37E-Gfus, would produce MPs truncated at the C terminus by 4 amino acids and fused with GFP65T; and would lack an intact CP gene.

Fluorescence microscopy observations was performed on protoplasts inoculated with transcripts of LQwt-Gfus, LQ238A-Gfus, LQ37A-Gfus, LQ37T-Gfus, and LQ37E-Gfus. Inoculated protoplasts were cultured in the presence of 30 μg of actinomycin D per ml and were fixed as described previously (18). Fluorescence micrographs were obtained with 400-speed Fujichrome slide film, using OLYMPUS BX60 with epifluorescence attachment and a U-MWIBA filter cube containing a BP 460-490 excitation filter, a DM505 dichroic mirror, and BA-510-550 emission barrier filter. The micrographs of the ring panels in Fig. 8 were photographed with 400-speed Fujichrome slide film and Leica MZ12 with epifluorescence attachment and a GFP plant filter set containing a 470- to 520-nm excitation filter, a 505-nm LP dichroic mirror, and a 525- to 575-nm emission barrier filter.

RESULTS

Phosphoamino acid and 2-D analysis of MP labeled in vivo. Phosphoamino acid analysis of ToMV MP was carried out on wild-type ToMV MP produced in infected protoplasts (44). [32P]-labelled MP was produced following inoculation of ToMV RNA into BY-2 protoplasts, metabolic labeling of the protoplasts with [32P]orthophosphate (44), and immunoprecipitation of the MP with anti-MP antisera (28). Labeled MP was recovered following SDS-PAGE, subjected to hydrolysis with 6 M HCl, mixed with standard phosphoamino acids, and subjected to TLC-electrophoresis (4). The results of these studies revealed that only serine is phosphorylated, as shown in Fig. 2, but we could not conclude how many serine residues are phosphorylated.

Assigning one of the phosphorylation sites by using previously described ToMV MP mutants and significance of the phosphorylation. Samples of 2-D analysis of the phosphopeptides of the MP were produced following trypsin digestion of phosphorylated MP. As shown in panel L of Fig. 3, three radioactive peptides (spots 1 to 3) appeared in the 2-D analysis; the simplest interpretation of these data is that three tryptic peptides are phosphorylated. However, this explanation was inaccurate, as shown below.

To facilitate the following discussion, potential tryptic peptides that contain serine residues were predicted from the known amino acid sequences of the MP and are shown in Fig. 1C. Each peptide is designated by increasing distance from the N-terminus as peptides 1 to 39 (Fig. 1C).

We previously isolated and characterized several ToMV MP mutants (31), and these mutants were evaluated for susceptibility to phosphorylation. Some of the amino acid differences compared with wild-type ToMV were predicted to give rise to different patterns of tryptic phosphopeptides.

ToMV-2a is a strain which overcomes the tomato Tm-2 resistance gene, a gene whose function inhibits cell-to-cell movement of tobamoviruses (5, 46). Three amino acid differences were found in the MP of this strain: lysine to glutamic acid at residue 130 (peptide 18), serine to arginine at residue 238 (peptide 38), and lysine to glutamic acid at residue 244 (peptide 38). The last two amino acids are involved in the resistance-breaking trait (46). When the MP of this strain was used for phosphopeptide analysis and compared with wild-type ToMV, it gave a distinct pattern (panel 2a of Fig. 3); the MP of 2a gave a distinct pattern (panel L of Fig. 3); the MP of 2a gave a single spot (spot 4), while wild-type MP gave three spots (spots 1 to 3). The spots were superimposed on each other in the 2-D analysis of mixed samples (panel L + 2a of Fig. 3). It was confirmed that the sole phosphopeptide spot (spot 4) in the 2a MP had the same mobility as one of the three spots of the wild-type protein (spot 2).

Since peptide 18 in ToMV-2a does not contain a serine residue, we concluded that peptide 38 must explain the differences in the phosphopeptide maps. Ser 238 is the only serine residue in peptide 38, and ToMV 2a lacks serine at this location; therefore, it was expected that the analysis would reveal two spots rather than one. The observation of a single spot was resolved when it was noted that in wild-type MP, the amino acid sequence around Lys 244, a predicted site for trypsin digestion, is amino terminal to a glutamic acid residue. As pointed out by others (4), trypsin is unable to react in a stoichiometric manner with lysine or arginine when located N-proximal to negatively charged residues, including glutamic

![FIG. 3. 2-D analysis of tryptic peptides of in vivo-phosphorylated MP. In vivo 32P-labeled MP was immunoprecipitated with anti-MP antisera, gel purified, and subjected to trypsin digestion. The trypsinized peptides were lyophilized and spotted onto TLC plates. Electrophoresis was carried out in the first dimension (horizontal) and was followed by chromatography in the second dimension (vertical) (4). (Left) Phosphopeptide map of MP of wild-type ToMV (L); (middle) map of ToMV 2a; (right) a mixed sample of L and 2a phosphopeptides. Arrowheads indicated phosphopeptide spots; arrowheads of wild-type ToMV are labeled 1, 2, and 3; arrowhead of ToMV 2a is labeled 4. The right panel indicates that one phosphopeptide spot (arrowhead 2) of the wild-type MP has the same mobility as the sole spot (arrowhead 4) of ToMV 2a protein.](http://jvi.asm.org/Downloaded from http://jvi.asm.org/cgi/content/doi/2017/00000000/00000000)
acid and aspartic acid. Thus, it would appear that wild-type MP was partially cleaved to produce two radioactive tryptic peptides, peptide 38 and peptide 38+39, both of which include Ser 238. (The abbreviation 38+39 indicates an undigested product.) Thus, the loss of Ser 238 alone could have caused the disappearance of two radioactive peptide spots of peptides 38 and 38+39.

Peptide 39 alone was not detected in the analysis, and we therefore presumed that no other serine residues in the C-terminal 31 amino acids were phosphorylated, including Ser 257, 261, and 263 (44). Of these, we excluded Ser 263 as a possible phosphorylation site, since truncation of 3 amino acids from the C terminus, including this serine, did not abolish the susceptibility to phosphorylation (44). This fact and the data in the next section have shown that we cannot assign all phosphorylation residues to the C-terminal region, in contrast to earlier reports (8, 44).

To verify that Ser 238 is phosphorylated but Ser 257 and 261 are not, we constructed three separate mutants of ToMV, in which Ser residue 238, 257, or 261 was changed to alanine (A); these mutants are referred to as LQ238A, LQ257A, and LQ261A, respectively. Transcripts of the respective plasmids were inoculated into BY-2 protoplasts, and 32P-labeled MP was analyzed as above. The results of these studies showed that LQ257A and LQ261A gave the same phosphopeptide map as did wild-type MP, while LQ238A gave a single spot, as did the 238 MP (data not shown). We therefore concluded that Ser 238 is a phosphorylation site of ToMV MP. Because the MPs of 238 and LQ238A are phosphorylated in vivo, it was concluded that a second phosphorylation site(s) is not found within the C-terminal region.

We also conducted phosphorylation analysis with Ls1, a temperature-sensitive mutant with mutation of cell-to-cell movement (32, 35) and Lt1b, a resistance-breaking strain which overcomes the Tm-2 gene for resistance (30). Both MPs gave phosphotryptic peptides identical to those of wild-type MP. From this analysis, we excluded three peptides as being phosphorylated: peptide 22, where a proline-to-serine change of Ls1 is located, and peptides 10 and 19, where a cysteine-to-phenylalanine and a glutamic acid-to-lysine substitution occurs, respectively.

To determine the significance of the phosphorylation of Ser 238 with respect to MP function, we compared the virulence of phenylalanine and a glutamic acid-to-lysine substitution of Ls1 is located, and peptides 10 and 19, where a cysteine-to-phenylalanine and a glutamic acid-to-lysine substitution occurred, respectively.

In vivo phosphorylation of alanine mutants. Transcripts of wild-type construct (W3), LQ238A, LQ18A238A, LQ37A238A, LQ75A238A, and LQ89A238A were inoculated into BY-2 protoplasts. Each MP was immunoprecipitated with anti-MP antiserum and subjected to SDS-PAGE (10% polyacrylamide). Lane Mock contains immunoprecipitate from uninfected protoplasts. Radioactive bands were detected by a Fuji Image Analyzer. The bottom panel is a result of Western analysis of MP accumulation in the respective protoplasts with anti-MP antibody as described previously (44). Possible phosphorylation sites. Based on such criteria, we focused on several serine residues as candidates for phosphorylation: Ser 18, Ser 37, Ser 75 and Ser 89 (Fig. 1C). Peptide 10 (Fig. 1C), which includes Ser 75, was excluded as discussed above. This amino acid was, however, considered to be a good control for our studies.

Alanine residues were introduced by site-directed mutagenesis in place of the residues listed above in the background of mutant LQ238A. Five constructs were made and are referred to as LQ18A238A, LQ37A238A, LQ75A238A, and LQ89A238A, respectively. Designations use the number of the amino acid that was mutated to alanine.

The four mutant MPs were assayed for in vivo phosphorylation by inoculating transcripts of the viral cDNAs that contained mutant MP into BY-2 protoplasts. Figure 4 shows that only LQ75A238A did not show a radioactive MP band whereas wild-type MP, LQ238A, and each of the other mutants were labeled, indicating that substitution at Ser 37 eliminated MP phosphorylation in spite of accumulation of each MP (Fig. 4, bottom). Radioactive MP of LQ37A238A was not detected even when the gel was overexposed more than 10-fold. Thus, it was concluded that Ser 37 is the second site of phosphorylation of the MP.

Effect of mutation of Ser 37 to Ala or Thr on phosphorylation of MP. To determine the effect of mutagenesis of Ser 37 on phosphorylation of MP, we constructed mutant LQ37A. MP phosphorylation produced by mutants LQ37A, LQ37A238A, and LQ238A as well as wild-type ToMV were analyzed. As shown in Fig. 5, LQ37A MP is produced in protoplasts but is not phosphorylated, even though the MP retains Ser 238.

To eliminate the possibility that other mutations in MP were responsible for the lack of phosphorylation in LQ37A, the serine codon AGU was introduced at residue 37, creating a mutant named Q37S(AGU); the original serine codon was UCA. MP produced from LQ37S(AGU) was phosphorylated at a similar level to wild-type MP (data not shown). The mutants LQ37A and LQ37A238A were assayed for the ability to potentiellate cell-to-cell movement of ToMV. Transcripts of each cloned cDNA were inoculated into the necrotic-lesion host, N. tabacum cv. Xanthi-nc; however, neither mutant caused lesions (data not shown). Inoculation into systemic hosts of ToMV, including N. tabacum cv. Samsun and tomato plants (Lycopersicon esculentum Mill.GCR 26 [30]), also gave negative results;
i.e., there was no systemic infection of those plants. Other studies showed that both mutants caused necrotic local lesions on plant line 2005, a transgenic MP(1) local-lesion tobacco host (reference 14 and data not shown). In this host, movement function was complemented by expression of the MP transgene. These data support the conclusion that both mutants are normal except in cell-to-cell movement.

We considered the possibility that the kinase that phosphorylates MP belongs to a group of serine/threonine protein kinases (37) that would phosphorylate a mutant MP in which Ser 37 was mutated to threonine (Thr; T). The MP was mutated to construct LQ37T and LQ37T238A, and experiments were conducted to determine if mutant MPs were phosphorylated. As shown in Fig. 5, LQ37T and LQ37T238A were phosphorylated at the same level as wild-type MP and LQ238A. Approximately equal amounts of each MP was detected in protoplasts by pulse-labeling with [35S]methionine-[35S]cysteine at 8 h p.i. for 10 min, and [35S]-labeled MP bands were detected by a Fuji Image Analyzer.

Mutants LQ37T and LQ37T238A were also assayed for their ability to potentiate cell-to-cell movement of infection. Transcripts of cloned cDNA were inoculated into both N. tabacum cv. Xanthi-nc and Samsun; neither mutant caused local lesions or symptoms of systemic disease (data not shown). Both mutants caused necrotic local lesions on plant line 2005 (data not shown). It was therefore concluded that the MP mutants LQ37T and LQ37T238A were phosphorylated but were defective in cell-to-cell movement.

Effect of mutation to Asp and Glu at Ser 37. To determine if substitution of aspartic acid or glutamic acid for Ser 37 could mimic the effect of a negatively charged, phosphorylated form of Ser 37, we constructed mutants LQ37D and LQ37E. When the respective mutants were inoculated into protoplasts, MPs were produced but were not phosphorylated (data not shown). To check whether mutants LQ37D and LQ37E possess cell-to-cell movement function, transcripts of the respective cloned cDNAs were inoculated into N. tabacum cv. Xanthi-nc and plant line 2005 (data not shown). Neither mutant caused lesions on N. tabacum cv. Xanthi-nc, while both mutants caused necrotic local lesions on plant line 2005. These studies demonstrated that replacing Ser 37 with a negatively charged amino acid was not sufficient to restore phosphorylation of Ser 238 or the function of the mutant MP.

Distribution of wild-type and mutant MP-GFP fusion proteins in protoplasts. Recently it was reported that MPs of tobamoviruses are associated with plasmodesmata (18, 19), elements of the cytoskeleton (18, 27), and ER (19). It has been suspected that these localizations of MP are closely related to the movement function itself or to its targeting route to the plasmodesmata (6, 19, 36).

To gain insight into the role of phosphorylation and association of MP with the cytoskeleton (18), viruses that produce mutant MPs such that the GFP were genetically fused with the C termini of the mutants. Fusion proteins are referred to as 37A MP-GFP, 37T MP-GFP, 37E MP-GFP, 238A MP-GFP, and wild-type MP-GFP, respectively. Viruses which produced MP-GFP fusions were named LQ37A:Gfus, LQ37T:Gfus, LQ37E:Gfus, LQ238A:Gfus, and LQwt:Gfus, respectively.

Figure 6 shows the typical distribution of each MP-GFP in infected protoplasts between 9 and 24 h postinoculation (p.i.).
At least 50 protoplasts were observed at each time and classified according to the localization of fluorescence (data not shown). Wild-type MP-GFP showed punctate fluorescent structures (dots) at 6 to 9 h p.i., irregular fluorescent structures between 9 and 12 h p.i., and fluorescent patch and filamentous structures after 12 h p.i. (Fig. 6). Wild-type MP-GFP was also observed in plasmodesmata (data not shown). Similar observations were made with MP-GFP of TMV and Ob by Heinlein et al. (18, 19). Localization of 238A MP-GFP was less pronounced than that of the wild-type counterpart but was quite similar in overall appearance (Fig. 6).

The mutant 37A MP-GFP did not show a distinct localization between 6 and 96 h p.i. (Fig. 6); mutant 37A MP-GFP showed low levels of dispersed fluorescence with no evidence of localization even at later stages of infection (Fig. 6), even though the MP was synthesized to nearly the same level as wild-type MP (data not shown).

Mutant 37T MP-GFP showed a pattern of fluorescence like that of wild-type MP-GFP (Fig. 6), with a delayed time course. Infection by virus that produced 37E MP-GFP produced punctate fluorescence, irregular fluorescent structures, and fluorescent patches and filamentous structures (Fig. 6); however, the structures appeared to be disordered compared with those of wild-type MP-GFP (data not shown). 37E MP-GFP showed filamentous structures in a low percentage of protoplasts (<10%) (Fig. 6) at later times after inoculation than those seen for wild-type MP-GFP. These data may indicate that a negative charge at residue 37 plays a role in targeting the MP to microtubules. Since the mutants LQ37T and LQ37E are defective in cell-to-cell movement function, we concluded that these mutations result in the loss of some activity(s), such as ssRNA binding activity, capacity to increase the size exclusion limit of plasmodesmata, or the ability to alter the stability of the MP.

**Substitution at serine 37 reduce the stability of the MP in vivo.** To examine whether there is a difference in the stability of wild-type and mutant MPs, pulse-chase experiments were performed. Protoplasts inoculated with virus that produced wild-type or mutant MPs were labeled for 10 min with $[^{35}S]$methionine-$[^{35}S]$ cysteine labeling mixture 8 h p.i. After 10 min, unlabeled methionine and cysteine were then added to the culture medium (see Materials and Methods) and equivalent amounts of protoplasts were harvested immediately or 1, 4, 16, or 28 h after labeling. Proteins were separated by SDS-PAGE, gels were scanned with a Fuji Image Analyzer, and the amounts of $[^{35}S]$MP were normalized to the density of MP bands at the initiation of the chase period. As summarized in Fig. 7, the 238A MP was as stable as wild-type MP through 28 h. In contrast, MP mutants 37A and 37E, neither of which is phosphorylated, were less stable than wild-type MP and 238A MP. MP mutant 37T, which is phosphorylated but does not facilitate cell-to-cell spread of infection, exhibited intermediate stability compared with wild-type MP and 37A MP. LQ37E MP was less stable than was LQ37A MP. These results indicate that phosphorylation and/or substitution at codon 37 had a significant effect of the stability of the MP.

**Comparison of fluorescent rings produced by MP-GFP.** We observed differences in the width of fluorescent rings produced by wild-type and mutant MP in MP(+) plants infected with viruses which produced MP-GFP fusion proteins. The cells that comprise the fluorescent rings showed filamentous structures, punctate fluorescence, irregular fluorescent structures, and fluorescent patches (18, 19) depending on the radial position of the cells inside the ring. Viruses were inoculated into wild-type *N. benthamiana* and plant line H$_{3}$ Nb-3, which is a transgenic MP(+) line derived from *N. benthamiana*. Wild-type virus LQwt:Gfus and LQ238:Gfus caused fluorescent rings on both wild-type *N. benthamiana* and plant line H$_{3}$ Nb-3, while other mutant viruses, LQ37A:Gfus, LQ37T:Gfus, and LQ37E:Gfus, caused fluorescent rings only on plant line H$_{3}$ Nb-3. We compared the diameters and widths of fluorescent rings produced by mutant viruses 3 days p.i. in plant line H$_{3}$ Nb-3. There was no significant difference in the diameters of fluorescent rings produced by wild-type and mutant viruses, but a difference in the widths of rings produced by wild-type and mutant viruses was observed (Fig. 8). A similar diameter of the ring reflects the cell-to-cell movement of wild-type and mutant viruses, all of which were equally supported by MP produced by the transgenic line H$_{3}$ Nb-3. Furthermore, we concluded that none of the mutant MPs interfered with the function of the wild-type MP in H$_{3}$ Nb-3. The fluorescence widths of the rings of LQwt:Gfus and LQ238:Gfus, which are both functional in cell-to-cell movement, were larger than those of defective mutants (Table 1). The width of the rings of LQ37A:Gfus was on average 13% of that of the rings of the wild type. LQ37T:Gfus and LQ37E:Gfus rings had widths of 32 and 28% of those of the rings of the wild type. These studies support the hypothesis that the MPs exhibits different degrees of stability.

**DISCUSSION**

We have identified two sites in the ToMV MP that are phosphorylated, Ser 37 and Ser 238, by a series of deductive studies that included $^{32}$P labeling of the protein in infected protoplasts and analysis of trypsin-digested protein. In vivo infectivity assays with mutants in which Ser 37 was changed to Ala 37 (mutant LQ37A) prevented phosphorylation and
showed that Ser 37 is essential for ToMV pathogenesis on tobacco and tomato plants. We could not conclude that phosphorylation per se was required for MP function since the LQ37T or LQ37T238A mutants, in which Ser 37 was changed to threonine, did not function even though these proteins were phosphorylated. Since Ser 37 could not be replaced with Thr, the results suggest that additional differences between Ser and Thr, such as differences in the side chain, affect the local or global structure of the protein. We concluded that Ser 37 in MP is essential for phosphorylation of the protein and that any substitution at this position alters the conformation of the MP, resulting in loss of function. Although analysis of MP mutants LQ37D and LQ37E supports the suggestion that phosphorylation of MP had a positive effect on the subfunction (intercellular localization) of the protein, placing a negatively charged amino acid (i.e., aspartic acid or glutamic acid) at position 37 did not restore the cell-to-cell movement function of the MP.

A comparison of MP amino acid sequences among the known tobamoviruses showed that Ser 37 is highly conserved among these viruses (data not shown). Accordingly, we also established a mutation in the MP of TMV (U1) with a serine-to-alanine substitution at residue 37. The MP of the mutant virus, referred to as U1Q37A, was also not phosphorylated in protoplasts (data not shown), and inoculation did not cause necrotic lesions on local-lesion tobacco hosts (data not shown). Thus, it is very likely that Ser 37 has a positive effect on phosphorylation of the protein, function of MP in cell-to-cell movement, and pathogenesis of tobamoviruses.

MP produced by mutants LQ37A was not phosphorylated. This may indicate that phosphorylation of Ser 37 in wild-type MP has a positive effect on phosphorylation at Ser 238. We suggest that sequential phosphorylation occurs in ToMV MP and that if phosphorylation does not occur at Ser 37, Ser 238 is also not phosphorylated. We propose that following phosphorylation at Ser 37, a conformational change in MP structure occurs, enabling access of the same or a second kinase to Ser 238.

Several different experimental procedures have been used to assay the phosphorylation of MP in lysates originating from MP(+) transgenic plants. Citovsky et al. reported a kinase activity in vitro that phosphorylates the endogenous and/or exogenously added MP (8). They argued that the kinase is tightly associated with the plant cell wall and used P30 (i.e., the MP) as substrate. The kinase activity which in vivo phosphorylates MPs at residues 37 and/or 238 is apparently different from that described by Citovsky et al., since the protoplasts used in our study have few or no cell walls. Furthermore, the phosphorylated sites (i.e., Ser 37 and 238) are apparently different from those described Citovsky et al. (8).

Citovsky et al. argued that phosphorylation of MP may represent a mechanism for the host plant to sequester MP in the mature tissue (8). In contrast, we propose that a kinase activity

![FIG. 8. Visualization of fluorescent rings produced by MP-GFP fusion viruses. Wild-type, LQ37A, and LQ37T MP-GFP fusion proteins were expressed from ToMV constructs. Fluorescence microscopy observations were made at 3 days p.i. in plant line H3Nb-3, a transgenic MP(+) line derived from N. benthamiana. The widths of rings are indicated by opposing arrows. Exposure times of micrographs of the ring produced by wild-type, LQ37A, and LQ37T MP-GFP fusion virus were 20, 272, and 32 s, respectively. Bars, 0.5 cm (top) and 0.2 cm (bottom).](http://jvi.asm.org/)

**TABLE 1. Comparison of fluorescent rings caused by MP-GFP fusion proteins**

<table>
<thead>
<tr>
<th>Name</th>
<th>Mutation in MP</th>
<th>Width of fluorescent ring (cm)</th>
<th>Diameter of fluorescent ring (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LQwt:GFP fus</td>
<td>None</td>
<td>1.23 ± 0.23 (10)</td>
<td>2.76 ± 0.42 (10)</td>
</tr>
<tr>
<td>LQ238A:GFP fus</td>
<td>Ser 23→Ala</td>
<td>1.17 ± 0.12 (10)</td>
<td>2.61 ± 0.47 (6)</td>
</tr>
<tr>
<td>LQ37A:GFP fus</td>
<td>Ser 37→Ala</td>
<td>0.16 ± 0.05 (13)</td>
<td>2.43 ± 0.37 (11)</td>
</tr>
<tr>
<td>LQ37T:GFP fus</td>
<td>Ser 37→Thr</td>
<td>0.39 ± 0.10 (13)</td>
<td>2.68 ± 0.46 (11)</td>
</tr>
<tr>
<td>LQ37E:GFP fus</td>
<td>Ser 37→Asn</td>
<td>0.35 ± 0.10 (14)</td>
<td>2.73 ± 0.59 (8)</td>
</tr>
</tbody>
</table>

*a* The MP(+) plant line H3Nb-3 was inoculated with in vitro transcripts derived from the viral constructs, and the diameters and widths of fluorescent rings produced by MP-GFP fusion viruses were measured at 3 days p.i.

*b* Mean ± standard deviation. Numbers in parentheses are the number of replicate determinations.
acts in the opposite way and activates the cell-to-cell movement functions of the MP.

Several researchers have reported that MP is localized to elements of the cytoskeleton (18, 27) in plant protoplasts. Heinlein et al. reported that during virus infection the MP-GFP fusion protein produces filamentous structures, which coalign with microtubules in BY-2 protoplasts. Based upon this information, we established ToMV mutants that produce fusion proteins, 37A MP-GFP, 238A MP-GFP and 37E MP-GFP, as well as wild-type MP-GFP. We observed time-dependent changes in fluorescent structures in protoplasts infected with viruses expressing functional MP; these include punctate structures (dots) (Fig. 6). Based on the recent report of Heinlein et al. (19), it is likely that the punctate structures are associated with cortical ER. While no apparent fluorescent structures were observed with virus that produced 37A MP-GFP, mutants LQ37T MP-GFP and LQ37E MP-GFP showed filamentous structures during late stages of infection (data not shown). These data suggested the possibility that a negative charge at position 37 could induce localization of MP-GFP on microtubules even though LQ37T and LQ37E are nonfunctional. It is also possible that LQ37T and LQ37E cannot execute a cell-to-cell movement function due to the delay in MP distribution or that these mutants have lost stability of the MP.

Pulse-chase experiments revealed that there are significant differences in the stabilities of the wild-type and mutant proteins. In these studies, carried out in the presence of actinomycin D, the mutant MP of LQ238A was as stable as wild-type MP. MPs of LQ37A and LQ37A238A, which were not phosphorylated, were less stable than wild-type MP, while MPs of LQ37T and LQ37E were intermediate in stability at early times in infection. These results suggest the possibility that phosphorylation of Ser 37 in MP has a positive effect on stability per se and is required for continual changes in higher-order structures of MP that alter the cell-to-cell movement functions of the protein.

Mutant LQ18A238A and LQ75A238A MPs were phosphorylated, but the level of phosphorylation was 26 and 68% of that of wild-type MP, respectively (Fig. 4). It seems that the phosphorylation level at serine 37 in MP was decreased by the replacement of serine 18 or serine 75 with alanine. These mutations in LQ18A238A and LQ75A238A MPs also showed 56 and 69% as much accumulation of each MP, respectively as did the wild type. This indicates that a decrease in the MP phosphorylation level is also linked to instability of the MP. Mutants LQ18A238A and LQ75A238A were assayed for the ability to undergo cell-to-cell movement. These mutant viruses were inoculated into a necrotic-lesion host, N. tabacum cv. Xanthi-nc. However, local lesions of the mutants LQ18A238A and LQ75A238A were smaller than those of the wild type and LQ238A. Taken together, these data also support the idea that susceptibility of phosphorylation of each MP is associated with its stability and leads to efficient cell-to-cell movement.

As a result of these studies, we concluded that the presence of serine at position 37 as well as phosphorylation of serine 37 is required for function of the MP in cell-to-cell movement of TMV infection. These studies showed that phosphorylation affects the stability of MP and its intracellular localization. Further elucidation of the mechanism of cell-to-cell movement of TMV requires further studies to determine whether phosphorylation of serine 37 affects specific functions of the MP, including the binding of the MP to single-stranded nucleic acids and gating of plasmodesmata.

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REFERENCES