Phosphorylation of Viral RNA-dependent RNA Polymerase and Its Role in Replication of a Plus-strand RNA Virus

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Central to the process of plus-strand RNA virus genome amplification is the viral RNA-dependent RNA polymerase (RdRp). Understanding its regulation is of great importance given its essential function in viral replication and the common architecture and catalytic mechanism of polymerases. Here we show that Turnip yellow mosaic virus (TYMV) RdRp is phosphorylated, when expressed both individually and in the context of viral infection. Using a comprehensive biochemical approach, including metabolic labeling and mass spectrometry analyses, phosphorylation sites were mapped within an N-terminal PEST sequence and within the highly conserved palm subdomain of RNA polymerases. Systematic mutational analysis of the corresponding residues in a reverse genetic system demonstrated their importance for TYMV infectivity. Upon mutation of the phosphorylation sites, distinct steps of the viral cycle appeared affected, but in contrast to other plus-strand RNA viruses, the interaction between viral replication proteins was unaltered. Our results also highlighted the role of another TYMV-encoded replication protein as an antagonistic protein that may prevent the inhibitory effect of RdRp phosphorylation on viral infectivity. Based on these data, we propose that phosphorylation-dependent regulatory mechanisms are essential for viral RdRp function and virus replication.

The reversible phosphorylation of cellular proteins provides a means to induce rapid and profound changes in the function or stability of a protein, and is recognized as a major process by which many aspects of cell biology are regulated. In the field of plus-strand RNA viruses, the largest class of viruses (1) that includes significant pathogens of humans, animals, and plants, there is growing evidence that phosphorylation of viral proteins plays an important role in regulating viral replication. The replication of plus-strand RNA virus genomes depends upon assembly of an intricate replication complex comprising both virus and host components (2). Within this complex, the viral RNA-dependent RNA polymerase (RdRp) plays a pivotal role in the viral infection process, catalyzing synthesis of new viral RNA genomes from the original infecting RNA in a two-stage process. The viral genomic plus-strand RNA first acts as a template for the synthesis of a complementary (minus-strand) RNA, which in turn directs synthesis of progeny plus-strand RNA. During replication, a large excess of plus- over minus-strands is produced, but the molecular mechanisms regulating this observed asymmetry and its temporal control are still largely unknown (3).

Several viral RdRps are now known to be phosphoproteins (4–7), and there is evidence to suggest that phosphorylation may regulate some aspects of their function. In Dengue virus type 2 and Cucumber mosaic virus, it was proposed that RdRp phosphorylation may regulate its interaction with other viral proteins during replication complex assembly (4, 6). Recently, new data have indicated that phosphorylation of Hepatitis C virus RdRp by a cellular kinase may be required for viral replication (7). Analysis of viral RdRp phosphorylation is especially important given the essential function of this enzyme in viral replication and the common architecture and mechanism of polymerase catalysis (8).

Here we address this question by studying phosphorylation of the RdRp of the tymovirus Turnip yellow mosaic virus (TYMV). The genomic RNA of TYMV, a plant-infecting member of the alphavirus-like superfamily of plus-strand RNA viruses (9), encodes two nonstructural proteins of 69 and 206 kDa (206K), the coat protein (CP) being expressed from a sub-genomic RNA. 206K, the only viral protein required for TYMV RNA replication, shares considerable sequence similarity with replication proteins of other plus-strand RNA viruses and contains domains indicative of methyltransferase, proteinase, NTPase/helicase, and RdRp activities. Self-cleavage of 206K generates an N-terminal 140K protein and a C-terminal 66K

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protein encompassing the RdRp domain (10). Both 140K and 66K are essential for TYMV RNA replication (11), and assembly of TYMV replication complexes, which are located at the periphery of chloroplasts, depends on interactions between the RdRp 66K and the membrane-bound 140K (12, 13).

We reported previously that TYMV RdRp is phosphorylated when expressed in a baculovirus/insect cell system (5), but the significance of this post-translational modification in the TYMV life cycle was not investigated. In this study, we demonstrate that TYMV RdRp is phosphorylated in plant cells, when expressed both individually and in the context of viral infection. Using a comprehensive biochemical approach, including metabolic labeling, peptide separation, and mass spectrometry analyses, two phosphorylation sites were mapped within an N-terminal PEST sequence and two others within the highly conserved palm subdomain of RNA polymerases. Systematic mutational analysis of the corresponding residues was used to investigate the correlation between RdRp phosphorylation, assembly of replication complexes, and viral infectivity. The results obtained support the idea that phosphorylation-dependent regulatory mechanisms are essential for viral RdRp function and virus replication.

**EXPERIMENTAL PROCEDURES**

**Plasmid Constructions**—Plasmid E17, from which full-length TYMV infectious transcripts can be obtained, plant expression vectors pΩ-66K, pΩ-140K, pΩ-206K, pΩ-CP, and pΩ-EGFP-140K and yeast two-hybrid vectors 2P-140K(555–1259) and pGad-66K were described previously (12–14). Mutations were introduced by PCR-mediated site-directed mutagenesis in plasmid E17, verified by sequencing, and subcloned in pΩ-66K and pGad-66K.

**In Vitro Transcription and Translation**—Synthesis of capped *in vitro* transcripts, *in vitro* translation reactions of viral RNA, or *in vitro* transcripts in rabbit reticulocyte lysate and their analysis by SDS-PAGE were performed as described (14). DNA fragments encoding WT or mutant 66K under the control of a T7 promoter were generated by PCR as described (10) and were transfected between 2 and 6 times using various batches of *in vitro* transcripts. Total protein extraction from protoplasts, SDS-PAGE, and immunodetection using anti-66K, anti-CP or anti-140K antibodies was performed as described (12, 13) except that 10% acrylamide, 0.13% bis-acrylamide was used to improve separation of the phosphoisoforms of 66K. Quantitation of viral proteins was performed by scanning blots from at least three independent replicate experiments, using serial dilutions of protein extracts and ImageJ software (National Institutes of Health).

**Expression of His-66K in Insect Cells, Metabolic Labeling, and Purification**—Spodoptera frugiperda cells were infected with recombinant baculovirus Bac8AF as described (5) using a mixture (1:1) of Grace and SF900-II (Invitrogen) culture media. *In vivo* metabolic labeling was performed by adding 62.5 μCi/ml [32P]orthophosphate to the growth medium 24 h post-infection. After a 24-h labeling period, the His-66K was purified by Ni2+-nitrilotriacetate affinity chromatography as described (5).

**Trypsin Digestion and Reverse-phase Chromatography**—Purified His-66K (~60 pmol) was reduced, alkylated, and digested with sequencing grade trypsin (Roche Applied Science) (5). The sample was loaded onto a reverse-phase HPLC column (5 μm, 250 × 1 mm, RP18, Spheri5; PerkinElmer Life Sciences) and washed with solvent A (0.1% trifluoroacetic acid). Peptides were separated by elution with a gradient consisting of 0–40% solvent B (0.1% trifluoroacetic acid in 90% acetonitrile) over 60 min, and 40–60% solvent B over 10 min, followed by isocratic elution with 100% solvent B. The flow rate was 50 μl/min, and peptide elution was monitored by UV absorbance at 214 nm. Fractions were collected manually, and aliquots were spotted on 3MM Whatman paper, and 32P radioactivity was detected by autoradiography.

**Mass Spectrometry (MS) Analyses**—HPLC fractions displaying radioactive content were mixed (1:1, v/v) with 2.5-dihydroxybenzoic acid (Aldrich) as a matrix and analyzed on a MALDI-TOF mass spectrometer (Voyager STR, Applied Biosystems) as described (5). To confirm peptide phosphorylation, the ions were selected for PSD analysis (15). For tandem mass spectrometry studies, HPLC fractions were dried completely, and peptides were resuspended in methanol/H2O/formic acid (12:7:1) and analyzed using a nano-ES Q-TOF I instrument (Micromass). The collision energy used for peptide fragmentation, adapted to each peptide, was typically between 18 and 40 eV. Tandem mass spectra of multiply charged parent ions were deconvoluted in monoprolonated ion fragments using MaxEnt3 software (Micromass).

**Transfection of Arabidopsis Protoplasts, Protein Extraction, SDS-PAGE, and Immunodetection Analyses**—Protoplasts of Arabidopsis thaliana were prepared and transfected as described (13). Each viral mutant was transfected between 2 and 6 times using various batches of *in vitro* transcripts. Total protein extraction from protoplasts, SDS-PAGE, and immunodetection using anti-66K, anti-CP or anti-140K antibodies was performed as described (12, 13) except that 10% acrylamide, 0.13% bis-acrylamide was used to improve separation of the phosphoisoforms of 66K. Quantitation of viral proteins was performed by scanning blots from at least three independent replicate experiments, using serial dilutions of protein extracts and ImageJ software (National Institutes of Health).

**Immunoprecipitation, Protein Phosphatase Treatment, and Metabolic Labeling**—Immunoprecipitation of 66K from protoplast extracts was as described (10) except that immunoprecipitates were collected using Pansorbin (Calbiochem). Immunoprecipitates were either directly subjected to SDS-PAGE and blotted to nitrocellulose membranes, or resuspended in phosphate buffer and incubated with 400 and 800 units of a phosphatase (New England Biolabs) for 1 h at 30°C prior to electrophoresis; where required, 50 nm EDTA was added to inhibit the phosphatase activity.

For coimmunoprecipitation experiments, transfected protoplasts were harvested 48 hpt and were lysed by incubation in buffer D (50 mM Tris-HCl, pH 7.5, 50 mM KCl, 0.5 mM EDTA, 20% glycerol, 0.5% Lubrol W) at 4°C for 45 min. Samples were then clarified by centrifugation for 40 min at 20,000 × g and incubated overnight at 4°C in the presence of anti-140K antisera. The antigen-antibody complexes were precipitated with Pansorbin, washed twice with buffer D, and analyzed by immunoblotting as described above. The nitrocellulose membranes were probed successively with the anti-66K and anti-140K antisera as described previously (13) to allow dual-color detection of the viral proteins.

**In Vivo** metabolic labeling was performed by adding 100 μCi/ml of [32P]orthophosphate to phosphate-free culture medium 2 hpt. After the labeling period, the 66K protein was immunoprecipitated, subjected to SDS-PAGE, and blotted to...
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RdRp produced in insect cells is phosphorylated on serine and threonine residues (5). Despite the extensively documented ability of baculovirus expression systems to perform appropriate post-translational modifications of recombinant proteins, it was necessary to determine whether the TYMV 66K was also phosphorylated in plant cells, either when expressed individually or in the context of a viral infection.

Arabidopsis protoplasts were transfected with TYMV viral RNA or with the plasmid pβ-66K. This transient expression vector encodes TYMV 66K in a biologically active conformation, as demonstrated by its ability to trans-complement a defective viral mutant (12). Cell extracts were subjected to Western blot analyses using anti-66K antibody. As shown in Fig. 1A (lanes 1 and 2), the 66K produced in the course of viral infection appeared as a single band, consistent with previous reports (10). In contrast, an additional slower migrating form was detected in protoplasts transfected with pβ-66K, resulting in the formation of a double band (Fig. 1A, lanes 3 and 4).

To test whether the change in mobility of the 66K expressed in vivo was due to phosphorylation, 66K was immunoprecipitated 24 hpt. Immunoprecipitated 66K was incubated with 400 units (lane 2), 800 units (lane 3), or without (lane 4) λ phosphatase (Ppase) in the presence (+) or absence (−) of EDTA as indicated. The 66K protein was revealed by Western blot analysis. C, Arabidopsis protoplasts transfected with TYMV RNA, pβ-66K or water were labeled with [32P]orthophosphate, and samples were collected at 24, 48, and 72 hpt as indicated. Immunoprecipitated 66K was revealed by autoradiography (top) and Western blot analysis (bottom). D, autoradiography of [32P]-labeled 66K protein obtained by in vitro translation in RRL or WG extracts. E, autoradiography of two-dimensional tryptic peptide maps of in vivo [32P]-labeled 66K protein expressed in insect (left) or Arabidopsis (right) cells. The peptides were separated by thin layer electrophoresis in the 1st dimension and by chromatography in the 2nd dimension. The origin is marked by a black dot.

RESULTS

TYMV 66K Is Phosphorylated in Plant Cells—TYMV 66K RdRp produced in insect cells is phosphorylated on serine and threonine residues (5). Despite the extensively documented ability of baculovirus expression systems to perform appropriate post-translational modifications of recombinant proteins, it was necessary to determine whether the TYMV 66K was also phosphorylated in plant cells, either when expressed individually or in the context of a viral infection.

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To test whether the change in mobility of the 66K expressed individually in plant cells was because of phosphorylation, 66K was immunoprecipitated from Arabidopsis protoplast extracts and incubated with λ phosphatase. As shown in Fig. 1B, this treatment led to the disappearance of the slower migrating 66K protein (lanes 2 and 3), while control incubation in the absence of phosphatase (lane 1) or in the presence of an inhibitor (EDTA) did not (lane 4). This demonstrates that the mobility shift of the TYMV RdRp is indeed caused by phosphorylation of the protein in plant cells.

As a complementary approach, we performed in vivo metabolic labeling experiments. Arabidopsis protoplasts transfected with TYMV viral RNA or with pβ-66K were incubated for var-
ious times in medium containing $^{32}$P-orthophosphate, and 66K was purified by immunoprecipitation. Immunoblot analysis (Fig. 1C, bottom panel) confirmed adequate expression and immunoprecipitation of 66K, whereas $^{32}$P labeling was detected by autoradiography (top panel). When expressed individually, we observed that both the upper and lower bands of the 66K were radiolabeled (Fig. 1C, lanes 5, and 9). Interestingly, the 66K produced during viral infection was also radiolabeled (Fig. 1C, lanes 2, 5, and 8), although it appears as a single band with a migration similar to the nonphosphorylated form. This observation is consistent with our previous reports indicating that some phosphorylation events may not affect the electrophoretic mobility of the 66K protein (5). These metabolic labeling experiments therefore demonstrate that TYMV RdRp is produced in plant cells as a phosphorylated protein not only when expressed individually but also in the context of viral infection. Furthermore, the observation that the phosphorylation status of 66K differs when expressed individually and during viral infection (as revealed by electrophoretic mobility and radiolabeling; Fig. 1, A and C) indicates that 66K phosphorylation may occur on at least two sites, the one(s) that are responsible for the phosphorylation-dependent mobility shift being inhibited during the course of viral replication (see below).

Expression of 66K in in vitro cell-free translation systems revealed that, when synthesized in rabbit reticulocyte lysate (RRL), 66K appeared as a single band of high mobility (Fig. 1D, lane 1). In contrast, the phosphorylation-dependent mobility shift was clearly observed upon expression of 66K in wheat germ (WG) extract (Fig. 1D, lane 2) indicating that 66K can be phosphorylated in vitro.

To determine whether the phosphorylation pattern of 66K expressed in insect and in plant cells was similar, we compared their phosphopeptide maps. Arabidopsis protoplasts transfected with p12-66K were metabolically labeled in vivo, and $^{32}$P-labeled 66K was purified by immunoprecipitation. In parallel, in vivo $^{32}$P-labeled 66K produced in insect cells using a recombinant baculovirus was purified by affinity chromatography. Tryptic peptide two-dimensional analysis of the resulting phosphoproteins (Fig. 1E) revealed a complex pattern of spots, indicating that phosphorylation occurs on multiple sites both in insect and in Arabidopsis cells. Although it is obvious that some spots were specific for Arabidopsis cells, comparison of the overall phosphorylation pattern of 66K in both cell lines revealed a surprising qualitative similarity, despite the different origins of these cell types. Most importantly, 90% of the phosphate incorporated in insect cells was present in signals that were common to both cell types, indicating that apart from a few minor spots all 66K phosphopeptides detected in insect cells are also present in plant cells. This finding justified further characterization of TYMV RdRp phosphorylation target sites in the baculovirus-insect cell system, the high expression levels enabling further phosphopeptide analysis.

Identification of 66K Phosphorylation Sites by Mass Spectrometry Analyses—MS is a powerful tool in the characterization of post-translational modifications of proteins, including phosphorylation (15, 18–23). The identification scheme used to precisely identify phosphorylated residues is presented in Fig. 2A. $^{32}$P-Labeled 66K purified by affinity chromatography from insect cells was digested with trypsin, and the resulting peptides were resolved by reverse-phase high performance liquid chromatography (HPLC). The radioactive fractions (Fig. 2B), which contain a mixture of phosphorylated and nonphosphorylated peptides, were subjected to MALDI-TOF MS analyses to characterize the peptide content of each fraction. The ions observed in the MALDI spectra were assigned to tryptic peptides derived from 66K according to their mass (as presented for HPLC fraction 59 as an example in Fig. 2C). Peaks with a mass increment of 80 Da, the mass of a single HPO$_3$ moiety, were considered as candidate phosphopeptides. These peptides were further analyzed by PSD in MALDI-TOF MS (15). The presence of fragment ions with a mass loss of 98 Da, characteristic of dephosphorylation (−H$_2$PO$_4$) of the parent ion, confirmed their identification as phosphopeptides (as presented for peptide Ile$^{316}$–Lys$^{338}$ as an example in Fig. 2D). As summarized in Fig. 3A, the analysis of all radioactive fractions allowed the identification of the following phosphopeptides: D03-DWC-QEHLTHTSKP$^{315}$, 316-ANDYTAFDQSVQHGESVVLEAKL$^{338}$, 317-LPLJHFDPPAIPTPPVSTSVDPQK$^{79}$, 43-LDTHFLPPSRLLPLHDLPJAPITPPVSTSDPPQAK$^{78}$, and 79-ASPVPYYGEGFDSLAAFLPAHDPST$^{104}$ each carrying a single phosphate moiety. The peptide Ac-ghhhhhTPSASPThr$, which encompasses the N-terminal His tag, was observed as both mono- and bisphosphorylated.

The phosphopeptides were then subjected to nanoelectrospray tandem mass spectrometric analyses (nano-ES-MS/MS) to identify the phosphorylated residues (as presented for the peptide Ile$^{316}$–Lys$^{338}$ in Fig. 2E). The data obtained for peptides Ile$^{316}$–Lys$^{338}$, Leu$^{53}$–Lys$^{78}$, Leu$^{43}$–Lys$^{78}$, and Ala$^{79}$–Arg$^{104}$ allowed the assignment of phosphorylation target sites to Ser$^{326}$, Thr$^{64}$, and Ser$^{80}$ (Fig. 3A). The close proximity of the putative acceptor sites and the lack of discriminative product ions did not allow the identification of the phosphorylation site in peptide Asp$^{303}$Lys$^{315}$, which would be one of the residues Thr$^{310}$, Ser$^{312}$, or Thr$^{313}$. Neither could we assign experimentally the phosphorylation site within the N-terminal peptide. Because this peptide is specific to the His-tagged recombinant protein and has no equivalent in the virally encoded protein, its phosphorylation was not studied further.

The Phosphorylation Sites Are Located within an N-terminal PEST Sequence and within the Conserved Palm Subdomain of RNA Polymerases—Sequence comparisons and mutagenesis studies of RdRps from a wide range of RNA viruses have identified several conserved sequence motifs that are important for biological function (25–27), although crystal structures of several RdRps (28–31) have revealed that they share a common structure, resembling a right-hand structure with fingers, palm, and thumb subdomains, with other classes of polymerases. Such cumulative data support the hypothesis that RdRps share a common architecture and mechanism of polymerase catalysis (8).

The phosphorylated residues Thr$^{64}$ and Ser$^{80}$ are located in the N-terminal region of 66K (Fig. 3B), a finding that is in perfect accordance with previous results (5). The N-terminal region is highly divergent among the RdRps of plus-strand RNA viruses, but we reported previously that PEST sequences, con-
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A
1. [32P]-metabolic labelling of His-66K
2. Affinity chromatography on Ni-NTA resin
3. Trypsin digest of purified His-66K protein
4. Reverse-phase HPLC of tryptic peptides
5. MALDI-TOF MS analysis of radioactive fractions
6. PSD MALDI-TOF MS of candidate phosphopeptides
7. nanoES-MS/MS analysis of phosphopeptides

B
Time (min)
% radioactivity

Fraction number

C
Fraction 59

D
Peptide 316 - 338

E

[Image of mass spectra and peptide sequences]
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dential signals for protein degradation (32), were invariably identified in this region of tymovirus RdRps (5).

Remarkably, two phosphorylated peptides, Asp<sup>303</sup>–Lys<sup>315</sup> and Ile<sup>316</sup>–Lys<sup>333</sup>, map to the conserved palm subdomain of RdRps (Fig. 3B). The palm subdomain, which forms the catalytic core of viral polymerases, contains five highly conserved sequence motifs (A–E) found in all polymerases (26). Its core structure is particularly conserved across all classes of polymerases, and residues forming the catalytic active site are found within motifs A and C. Residues Thr<sup>310</sup>, Ser<sup>312</sup>, and Thr<sup>313</sup>, which constitute the possible phosphorylation sites within Asp<sup>303</sup>–Lys<sup>315</sup>, are located just N-terminal to motif A and are not highly conserved among viral RdRps. In contrast, Ser<sup>326</sup> is of particular interest because it is an integral part of motif A and corresponds to a residue that is strictly conserved in supergroup III RdRps and highly conserved in subgroup I polymerases (27). The evolutionary conservation of this residue from alphavirus to picornavirus RdRps, and its location in such a critical situation for polymerase biochemical function, suggests that its post-translational modification may play an important regulatory role in viral RNA replication.

To clarify the possible role of these phosphorylation target sites in the viral replication process, a comprehensive mutagenesis study was undertaken.

**Mutations of the 66K Phosphorylation Sites Affect Viral Infectivity**—To examine the importance of RdRp phosphorylation for viral infectivity, mutations affecting the phosphorylation target sites were introduced by site-directed mutagenesis in the context of an infectious TYMV cDNA clone (14). Phosphorylation-deficient mutants were produced by substituting target residues with alanine, while phosphorylation was mimicked by substituting serine and threonine with the negatively charged aspartic acid, shown in many previous studies to produce the electrostatic and steric effects of phosphorylation (23, 33–37). To examine the possibility that the kinase(s) involved is a serine/threonine protein kinase, we also generated mutants in which the serine and threonine acceptor sites were mutated to alanine, while the remaining serine/threonine residues were left unaltered. The serine/threonine protein kinase activity was assessed by cotransfection with an expression vector encoding SV40 large T antigen, which is known to stimulate the activity of several virus-encoded protein kinases (28).

**Mutations Affecting Thr<sup>310</sup>, Ser<sup>312</sup>, or Thr<sup>313</sup>**—The mutants were transfected into the infected leaf disks at an optimal concentration (31), and viral RNA was harvested at 10 days postinfection. The accumulation of viral RNAs was assayed by Northern blotting, and the corresponding full-length RNA transcripts in RRL demonstrated that the mutations had no influence on 206K expression and processing (data not shown). Equal amounts of *in vitro* transcripts were transfected into *Arabidopsis* protoplasts, and the ability of the various mutants to replicate in transfected cells was assayed. WT TYMV RNA and *in vitro* transcripts were included as positive controls in each experiment, while the G404R mutant, carrying a mutation within the ultra-conserved GDD motif in the polymerase catalytic domain, served as a negative control (11). Each mutant was transfected between 2 and 6 times using various batches of *in vitro* transcripts, and the accumulation of viral CP and plus-strand RNAs was assayed by Western and Northern blots, respectively (Fig. 4).

Mutations affecting Thr<sup>310</sup> or Ser<sup>312</sup> had only minor effects on the accumulation of viral products (Fig. 4, *lanes 1–7*). Comparable results were obtained upon transfection of T64A/S80A, T64D/S80A, and T64A/S80D double mutants (Fig. 4, *lanes 8, 10, and 11*). However, when combined in the T64D/S80D mutant, the phosphorylation-mimicking mutations resulted in a dramatic impairment of viral infectivity (Fig. 4, *lane 9*) because accumulation of CP and plus-strand RNA was reduced ∼10-fold compared with WT viral RNA transfected in parallel (cf. *lanes 9 and 12*).

Mutations affecting Thr<sup>310</sup>, Ser<sup>312</sup>, or Thr<sup>313</sup> had only minor effects on the efficiency of viral replication (Fig. 4, *lanes 14–23*).

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**FIGURE 2. Identification of 66K phosphorylation sites by mass spectrometry analyses.** *A*, scheme used for the identification of phosphorylation sites (for details, see “Experimental Procedures”). *B*, HPLC reverse-phase separation of His-66K tryptic peptides (*upper panel*) and detection of radioactivity in each fraction (*lower panel*). The arrow indicates fraction 59. C, MALDI-TOF MS of HPLC fraction 59. The mass of the peak at m/z 2615.27 is 80 Da higher than the calculated mass for the tryptic peptide Ile<sup>316</sup>–Lys<sup>333</sup>, indicating a candidate phosphopeptide. According to their mass, the other ions were identified as internal nonphosphorylated peptides as indicated above each peak. D, PSD MS of peptide at m/z 2615.30 from HPLC fraction 59. The peak at m/z 2516.80 is a diagnostic fragment ion, the mass loss of 98 Da being characteristic of dephosphorylation (H<sub>3</sub>PO<sub>4</sub>) of the parent ion. E, nano-ES Q-TOF tandem MS of MS of peptide at (M + H)<sup>+</sup> = 2615.30 m/z from fraction 59. Using collision-induced dissociation analysis, the peptide produced a clean ion series, which allowed both confirmation of the peptide as Ile<sup>316</sup>–Lys<sup>333</sup> and assignment of phosphorylation to Thr<sup>326</sup>. Fragment nomenclature is according to Ref. 24. The vertical *numbering below* the single-letter code sequence refers to y ions, i.e., charged C-terminal peptide fragments. The *vertical numbering above* the single-letter code sequence refers to b ions formed by charge retention on the N-terminal acylium fragments. The calculated masses based on the presence of a phosphoserine residue at position 326 are indicated, and the observed ions are shown in *boldface*. The scale on the y axis is expanded (3× from 0 to 1800 m/z and 1.5× above 1850 m/z) to improve visualization of key fragment ions y<sub>12</sub> (m/z 1309.71) and y<sub>13</sub> (m/z 1476.71), which, given the mass increment of 167 Da, are diagnostic of phosphorylation at Ser<sup>326</sup>. This assignment is consistent with the presence of satellite peaks formed by the loss of phosphate (y<sub>12</sub>H<sub>3</sub>PO<sub>4</sub>) on all of the y ions containing the phosphoserine residue (y<sub>13</sub> to y<sub>22</sub>).
In contrast, mutations affecting residue Ser\textsuperscript{326}, which is part of the conserved motif A, had pronounced effects on viral replication and accumulation of viral products. Mimicking phosphorylation of this residue in the S326D mutant resulted in complete inhibition of plus-strand viral RNA synthesis and CP accumulation (Fig. 4, lane 28). Substitutions of Ser\textsuperscript{326} for alanine or threonine also severely impaired viral infectivity. Upon infection with the nonphosphorylatable mutant S326A, CP and plus-strand RNA accumulation were reduced \(\times 4\) and \(\times 3\)-fold, respectively, compared with WT viral RNA transfected in parallel, while substitution of Ser\textsuperscript{326} for threonine led to a \(\times 10\)-fold reduction in their accumulation (Fig. 4, cf. lanes 26, 27, and 29).

To ensure that the defects in viral infectivity were not because of nucleotide changes in the RNA template, trans-expression experiments were performed, and trans-complementation was assayed by immunodetection of the viral CP. Mutant viral in vitro transcripts T64D/S80D, S326A, S326D, S326T, and G404R were transfected in Arabidopsis protoplasts together with the pΩ-66K expression vector encoding WT 66K protein. As a complementary approach, the defective viral transcript E17-stopΔ, which does not encode 66K (12), was provided as a template and was cotransfected with plasmid constructs expressing WT or T64D/S80D, S326A, S326D, or S326T mutant 66K proteins. The results obtained (Fig. 5) demonstrated that the defects in viral infectivity were caused by mutations of the 66K at the protein level, rather than changes in the RNA template.

The possibility that poor accumulation of T64D/S80D, S326A, and S326T viral RNA was caused by a lack of encapsidation was also ruled out as these mutants could systemically infect plants (data not shown), a process that is dependent upon virion formation (38).

Altogether, these results indicate that mutation of the identified phosphorylated residues Thr\textsuperscript{64}, Ser\textsuperscript{80}, and Ser\textsuperscript{326} severely inhibits TYMV infectivity and suggest that host kinases may exert profound control over viral replication by promoting phosphorylation of several of these residues.

**Mutations of the Phosphorylation Sites Do Not Affect the Ability of 66K to Interact with 140K**—66K is recruited to the replication complexes located at the periphery of chloroplasts through protein-protein interactions with the membrane-bound TYMV-encoded 140K protein (12, 13). Because the 140K interaction domain, mapped to residues 1–369 of 66K (13), contains the identified phosphorylation sites (Fig. 3B), we next tested whether the inability of certain
mutants to replicate efficiently was caused by a defect in the 66K-140K interaction.

First, yeast two-hybrid interaction assays were performed to evaluate whether the ability of 66K to interact with the C-terminal region of 140K (13) changed upon mutation of Thr^64, Ser^80, or Ser^326. All mutant proteins were found to retain WT levels of interaction with the 140K protein (Fig. 6A).

To ascertain whether this was also the case in plant cells, we examined the subcellular localization of 66K and 140K in transfected Arabidopsis protoplasts. We had shown previously that 66K is distributed in the cytoplasm when expressed individually but is recruited to the chloroplast envelope upon interaction with the membrane-bound 140K protein (12, 13). The expression vector pΩ-EGFP-140K, encoding the 140K protein fused to the EGF, was cotransfected with in vitro transcripts corresponding to the WT viral genome or to the mutants T64D/S80D, S326A, or S326T. Indirect immunofluorescence assays combined with the use of EGF autonomous fluorescence allowed simultaneous detection of 140K and 66K viral proteins (13).

Analysis of cotransfected cells by confocal laser scanning microscopy revealed no differences in 140K-dependent recruitment of mutant 66K proteins to the chloroplast envelope as compared with WT RdRp (Fig. 6B). Because the S326D mutation prevents viral replication, and thus localization of the corresponding protein could not be assayed in the context of a viral infection, pΩ-EGFP-140K was cotransfected with the plasmids pΩ-66K or pΩ-66K-S326D. Again, we observed no difference in the ability of the mutant 66K protein to be recruited to the chloroplast envelope by the 140K protein.

The physical interaction of the TYMV replication proteins in plant cells was confirmed by performing coimmunoprecipitation experiments. For this purpose, expression vectors encoding WT or mutant 66K protein were transfected in Arabidopsis protoplasts together with the pΩ-140K expression vector, and protein extracts were subjected to immunoprecipitation under non-denaturing conditions using the anti-140K antiserum (13). Western blotting analyses using the anti-66K and anti-140K antisera successively revealed that both the 66K protein and the two major proteins derived from the 140K protein, the 115K and 85K proteins (13), were present in the immunoprecipitates (Fig. 6C, lanes 2 and 10). Similar results were obtained when the 140K protein was expressed together with the T64D/S80D, S326A, S326D, or S326T 66K mutant proteins (Fig. 6C, lanes 8, 12, 14, and 16), therefore demonstrating that the mutant 66K proteins physically interacted with the 140K-derived proteins in plant cells.

These results therefore rule out the possibility that the altered replication of viral mutants T64D/S80D, S326A, S326D, and S326T is caused by a defect in the ability of mutant 66K to interact with the 140K protein.

Mutations of the Phosphorylation Sites Affect Different Steps of the Viral Replication Cycle — To gain further insights into the possible effects of these mutations in the viral replication cycle, we examined the accumulation of minus-strand RNA replication intermediate and 66K RdRp were assayed with selected viral mutants (Fig. 7).

Infection with T64D/S80D viral mutant led to a decrease in the accumulation of minus-strand RNA (Fig. 7, cf. lanes 2 and 5), whose extent was similar to that observed with plus-strand RNA (Fig. 4) indicating interference with RNA replication throughout the entire replication cycle. Interestingly, the amount of 66K detected appeared reduced more than 3-fold compared with WT (Fig. 7, cf. lanes 2 and 5), suggesting that the inability of the T64D/S80D mutant to replicate efficiently may relate to limiting amounts of 66K.

In cells infected by S326A or S326T mutants, we observed that the accumulation level of 66K was similar to WT levels (Fig. 7, cf. lanes 8, 9, and 11). Strikingly, we also observed no defect in minus-strand RNA accumulation as levels attained were similar to (S326T) or even exceeded by ~2-fold (S326A).
those accumulating in the course of a WT infection. The ability of both mutants to properly synthesize minus-strand RNA indicates that the defects in viral infectivity observed upon substitution of Ser^{326} (Fig. 4) are not caused by a major deficiency in polymerase catalytic activity, but rather that regulation of RNA synthesis is specifically affected.

**FIGURE 6.** Mutations of the phosphorylation sites do not affect the 66K/140K interaction. A, proteins containing an N-terminal Gal4 DNA binding domain fused to WT or mutant 66K protein, as indicated, were tested for interaction with the C-terminal region of 140K (residues 555–1259) fused to the Gal4 activation domain in the yeast two-hybrid system (13). Serial 5-fold dilutions of yeast cells were plated on both interaction-nonselective (−LW) and interaction-selective (−LWH) media. Quantitative β-galactosidase (β-gal) activity assays were performed, and the means (in Miller units) ± S.D. are indicated on the right. Typical interactions are shown along with positive (T+) and negative (T−) controls (13). B, Arabidopsis protoplasts were transfected with the expression vector pΩ-EGFP-140K together with WT or mutant viral in vitro transcripts or expression constructs encoding WT or mutant 66K protein as indicated. Transfected protoplasts were collected at 42 hpt and were processed for indirect immunofluorescence (IF) labeling using anti-66K antiserum and secondary antibodies coupled to AlexaFluor 594. Single protoplasts were observed by confocal laser scanning microscopy using sequential acquisition of EGFP fluorescence (green) and AlexaFluor 594 fluorescence (red). The merged images represent a digital superimposition of red and green signals in which areas of fluorescence colocalization appear yellow. Scale bars are 10 μm. C, Arabidopsis protoplasts were transfected with expression constructs encoding WT or mutant 66K protein, alone or in combination with the expression vector pΩ-140K encoding 140K protein, as indicated. Transfected protoplasts were collected at 48 hpt, and protein extracts were processed for immunoprecipitation using anti-140K antiserum under nondenaturing conditions (13). The immunoprecipitates (IP) and an aliquot of the protein extracts corresponding to 10% of the input sample (input) were sequentially revealed by Western blotting using anti-66K and anti-140K antisera and nitro blue tetrazolium-5-bromo-4-chloro-3-indolyl phosphate (purple) and Fast Red (red) substrates, respectively. The position of immunoglobulin (Ig) heavy chains is indicated.
The S326D mutation appeared to have the most drastic effects on the replication cycle, as neither the 66K protein nor minus-strand RNA replication intermediate was detectable (Fig. 7, lane 10). Providing the 66K protein in trans from the pQ-66K-S326D expression vector was insufficient to promote minus-strand RNA synthesis and to initiate the viral replication process (see Fig. 5B, lane 5, and data not shown), indicating that the lack of 66K protein does not account for the failure of the replication cycle to start. Therefore, it is likely that the S326D mutation interferes with replication before or at the onset of minus-strand synthesis, which consequently prevents the accumulation of plus-strand viral RNAs and encoded proteins.

Altogether, these results indicate that phosphorylation of TYMV RdRp is likely to regulate different steps of the viral replication cycle. They strongly implicate Thr64 and Ser80 in the accumulation level of 66K, while Ser326 appears involved in the control of RNA synthesis.

66K Phosphorylation Is Antagonized during Viral Infection by Expression of 140K—The difference in 66K phosphorylation when expressed individually or in the context of viral infection (Fig. 1, A and C) suggests that some virally encoded protein might suppress phosphorylation of the 66K protein at certain sites, in particular those whose phosphorylation is visible as the 66K mobility shift. Given the deleterious effect of 66K phosphorylation on viral replication (Fig. 4), we also anticipated that its phosphorylation might be antagonized during at least some steps of viral replication.

To address this question, we first aimed to identify the residue(s) responsible for the phosphorylation-dependent mobility shift of 66K. Single and double substitution mutants were expressed in in vitro translation extracts or in Arabidopsis protoplasts, and the mobilities of the corresponding mutant 66K were revealed by autoradiography (Fig. 8, A and B, lanes 2), while the T64D mutant displayed an upward mobility shift (Fig. 8, A and B, lane 3). Consistently, similar downward and upward shifts were observed in double mutants affecting both Thr64 and Ser80 (Fig. 8, A, lanes 8–11, and B, lanes 6 and 7). These results therefore identified Thr64 as the sole phosphorylation site causing the electrophoretic mobility shift of TYMV RdRp.
Next, we compared the electrophoretic mobility of 66K when expressed either individually, in the presence of 140K, or within the context of the 206K polyprotein precursor (Fig. 8C). Upon expression of the 206K polyprotein in Arabidopsis protoplasts, the phosphorylation-dependent mobility shift of 66K was undetectable (Fig. 8C, lane 3). Similarly, coexpression of 140K inhibited the mobility shift of 66K (Fig. 8C, lane 4), while coexpression of the CP as a control had no effect (lane 5). These results indicate that expression of the 140K replication protein antagonizes 66K phosphorylation at certain sites, in particular Thr<sup>64</sup>, phosphorylation of which is visible as the 66K mobility shift, and suggest that phosphorylation of the RdRp might be inhibited during viral RNA replication.

**Phosphorylation Status of the Identified Residues in the Course of Viral Infection—**To determine whether any of the identified residues become phosphorylated during the course of viral replication, viral RNA mutants in which phosphorylation target sites were individually mutated to alanine were transfected into Arabidopsis protoplasts, and in vivo metabolic labeling experiments were performed to detect 66K phosphorylation in the course of viral infection. Following purification of 66K by immunoprecipitation, equivalent amounts of 66K were analyzed by Western blotting, and their phosphorylation level was detected by autoradiography and quantified (Fig. 9). The T64A and S80A 66K mutant proteins were phosphorylated in vivo to levels similar to those obtained with the WT protein (lanes 2 and 3), indicating that Thr<sup>64</sup> and Ser<sup>80</sup> make only a minor contribution, if any, to the overall phosphorylation of 66K during the course of viral infection. These results are in perfect agreement with the data presented above (Fig. 8) and consistent with the infectivity assays (Fig. 4) indicating that constitutive phosphorylation of Thr<sup>64</sup> and Ser<sup>80</sup> is highly detrimental to the viral accumulation process.

In contrast, the phosphorylation level of T310A, T313A, and S326A mutants was significantly reduced compared with WT protein (Fig. 8, lanes 4, 6, and 7), indicating that Thr<sup>310</sup>, Thr<sup>313</sup>, and Ser<sup>326</sup> are indeed phosphorylated during viral multiplication.

**DISCUSSION**

Although well documented in negative-strand RNA viruses (39-41), the role of phosphorylation in regulating the activities and functions of nonstructural proteins in plus-strand RNA viral genomes is only now beginning to emerge (4, 6, 7, 42-45). In this study, we present a documented characterization of phosphorylation sites of a viral RdRp.

As the low abundance of the phosphorylated TYMV RdRp produced in plant cells precluded its biochemical analysis, characterization of the phosphorylated residues was performed by MS analyses of the phosphoprotein produced in insect cells. The tryptic peptide two-dimensional analysis (Fig. 1E) was of extreme importance in this rationale as it revealed that most of the phosphopeptides detected in insect cells were also present in plant cells. It is clear, however, that other significant phosphorylation events occur specifically in Arabidopsis cells, but those were not characterized in the present study.

The strategy used for determination of protein phosphorylation sites relied on a combination of approaches (Fig. 2A) that have proven useful previously (18-23); metabolic labeling of the His-tagged 66K phosphoprotein provided the advantage of the easy purification of the protein produced in cells with the high sensitivity of <sup>32</sup>P detection. Following trypsin digest, the peptides were separated by HPLC, and phosphopeptides-containing fractions were identified by autoradiography prior to MS analyses. This separation step aims at reducing the ion suppression phenomenon that may occur in complex peptide mixtures (46), therefore increasing the chance of detecting phosphopeptides. A combination of MALDI-TOF and PSD analyses (Fig. 2, C and D) performed on the selected radioactive mixtures was then used to identify the candidate phosphopeptides, whose sequence was finally determined using nano-ES-MS/MS (Fig. 2E). This strategy led to the identification of four phosphorylated residues in TYMV RdRp. Two of them correspond to previously described phosphorylated residues located in the N-terminal region of 66K (5), while two others correspond to previously unknown sites (Fig. 3).

The results obtained were subsequently borne out by experiments performed in plant cells. Mutagenesis of the identified phosphorylation sites in a reverse genetics system and analysis of their phosphorylation status by metabolic labeling confirmed that Thr<sup>310</sup>, Thr<sup>313</sup>, and Ser<sup>326</sup> are phosphorylated in the course of viral infection (Fig. 9). Analysis of the electrophoretic mobilities of the corresponding RdRp mutant proteins expressed in plant cells from an expression vector provided evidence that phosphorylation of Thr<sup>64</sup> occurs in plant cells when 66K is expressed individually (Fig. 8A) but that it is inhibited upon expression of the TYMV 140K.
Phosphorylation/dephosphorylation can induce rapid and profound changes in the function of a protein in several ways as follows. It can alter conformation of an enzyme active site. It can allow a protein to move from one subcellular component to another or mark it for destruction. It can also control protein-protein interactions by creating or eliminating docking sites for specific binding partners. During infection, viral RdRps act in combination with other viral and host factors to assemble replication complexes, to select template RNAs, and to initiate and maintain elongation of RNA synthesis. Thus, there are many ways in which phosphorylation of RdRp may affect its function.

Studying the effects of phosphorylation on protein function is a challenging task because of the substoichiometric and often labile nature of phosphorylation. To circumvent this problem, the biological function of RdRp phosphorylation was examined by substituting Asp or Ala residues for the phosphorylation target sites, respectively, mimicking the phosphorylated or nonphosphorylated status of Ser and Thr residues. Such an approach has been widely and successfully used in the literature (23, 33–37). The results obtained upon infection with the corresponding viral mutants strongly support the idea that RdRp phosphorylation is critical for TYMV viral replication and provide insights into the possible role(s) of phosphorylation in regulating the activities of RdRp function.

Unlike other plus-strand RNA viruses (e.g. Dengue virus type 2 and Cucumber mosaic virus), where phosphorylation of RdRp appears to be involved in regulating interaction with replication proteins (4, 6), our results (Fig. 6) indicate that, in TYMV, phosphorylation is likely to regulate some other aspects of RdRp function. However, we cannot exclude the possibility that assembly of TYMV replication complexes is regulated by phosphorylation at yet uncharacterized residues. Based on the detailed analysis of viral proteins and RNA products produced upon infection with TYMV mutants (Figs. 4 and 7), our working model proposes that phosphorylation is a dynamic process that may regulate at least two different aspects of RdRp function (Fig. 10).

Substitution of the phosphomimic Asp amino acid for Thr and Ser residues led to a strong defect in viral accumulation, affecting the entire replication cycle (Figs. 4 and 7). As those residues are located within a PEST sequence that is conserved among tymovirus RdRps (5), we therefore propose that phosphorylation of Thr and Ser may serve to regulate the accumulation level of 66K, the activation of the latent PEST signal controlling the metabolic stability of the protein (Fig. 10, box A). Mimicking Thr and Ser phosphorylation would thus lead to increased turnover of RdRp and compromise its accumulation. In such a situation, 66K would thus become a limiting component in the replication machinery, resulting in the inhibition of both minus- and plus-strand RNA synthesis. This hypothesis is consistent with the decreased accumulation of 66K observed upon infection with the T64D/S80D mutant (Fig. 7) and with the finding that mutation of these phosphorylation sites affects RdRp stability in vivo. The 66K protein was reported previously to be conjugated with ubiquitin in insect cells (5). Whether it is degraded through the ubiquitin-dependent proteasome pathway in plant cells is currently under study.

Because the hypophosphorylated mutant T64A/S80A replicates as efficiently as wild type (Figs. 4 and 7), and because phosphorylation of Thr and Ser was not detected in the course of viral infection (Figs. 8 and 9), phosphorylation of these sites does not appear to be strictly required for viral replication. Instead, we propose that phosphorylation of these residues in 66K might represent a host cell defense pathway aimed at inac-

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tivating this key viral protein. The inhibition of Thr\textsuperscript{64} phosphorylation observed during viral infection or upon expression of the TYMV 140K replication protein (Fig. 8) highlights the role of 140K as an antagonistic protein preventing the inhibitory effect of RdRp phosphorylation on viral infection (Fig. 10, box B). Whether the 140K directly inhibits the activity of the kinase(s) involved, protects 66K from phosphorylation by masking the phosphorylation sites, or prevents access of the kinase(s) to 66K because of 140K-mediated recruitment of 66K to the chloroplast envelope is currently unknown, and future experiments will allow us to distinguish between these not mutually exclusive possibilities. There is an increasing number of proteins whose stability is regulated by their level of phosphorylation and/or the presence of binding partner(s) (47). Our results support the idea that such conditional accumulation of TYMV RdRp may exert some control over viral infectivity, a finding that is, to our knowledge, without direct precedent.

Substitution of Ser\textsuperscript{326}, whose phosphorylation appears to account for \textasciitilde25\% of the overall phosphorylation of 66K during viral infection (Fig. 9), also caused severe defects in viral infectivity (Figs. 4 and 7). This finding is of particular interest given the location of this residue within the highly conserved motif A of the catalytic palm subdomain of polymerases, which suggests that its post-translational modification may play an important regulatory role in viral RNA synthesis. Although it may not be ruled out that the introduced substitutions caused a severe conformational change within the target protein, the fact that Ser\textsuperscript{326} mutants were all capable of interacting with 140K (Fig. 6) and that S326A and S326T mutant proteins retained the ability to synthesize RNA molecules (Figs. 4 and 7) strongly support the idea that the effects on viral replication observed upon Ser\textsuperscript{326} substitution are not caused by a gross defect in the structure of the protein, but rather that phosphorylation specifically interferes with RdRp function.

What may be the molecular mechanism(s) involved? In RdRps from which structural data are available, motif A is composed of a “β-strand, turn, α-helix” (48–50), and two strictly conserved Asp residues are involved in divalent cation coordination and selection of the incoming rNTP, respectively (8, 51). Ser\textsuperscript{326} residue is located at the end of the α-helix, and its proper orientation may therefore be required for the correct geometrical arrangement of polymerase, substrate molecules, and metal ions at the active site for catalysis. As phosphorylation adds extra negative charges to the protein and may also cause steric hindrance, it is conceivable that Ser\textsuperscript{326} phosphorylation might inhibit the correct assembly of the catalytic complex. The effect of phosphorylation on RdRp function may also be more complex, through conformational changes within the protein that may modulate its function(s) during various steps of the replication process (i.e. template or nucleotide selection, transition from initiation to elongation, processivity of the enzyme or product release) and that would therefore influence the efficiency, the ratio, or the timing of production of the different viral RNA species. In that respect, it has been reported recently that correct nucleotide incorporation, and hence RdRp fidelity, may be governed by a conformational change affecting one of the Asp residues within motif A, whose positioning is highly dependent on a network of interactions involving residues in motif B (51, 52) but also remote residues in the fingers domain (31, 53). Whether phosphorylation of Ser\textsuperscript{326} might have an impact on this complex interplay is currently unknown. Ultimately, in vitro biochemical assays using reconstituted replication complexes may help clarify the mechanism involved, while three-dimensional crystal structures of the two phospho-isoforms will be required to determine the precise effect of phosphorylation on their conformations.

Interestingly, both the hypophosphorylated mutant S326A and the phosphomimic mutant S326D mutant replicated less efficiently than WT (Figs. 4 and 7), suggesting that a balance between Ser\textsuperscript{326} phosphorylation and dephosphorylation is important for viral infectivity. We therefore propose (Fig. 10, box C) that post-translational modification of Ser\textsuperscript{326} is a dynamic process, perhaps acting as a molecular switch to regulate viral replication. As both S326T and S326A mutants presented a drastically altered plus- to minus-strand RNA ratio, one hypothesis is that phosphorylation of Ser\textsuperscript{326} may be involved in the regulation of strand asymmetry. Another possibility is that phosphorylation may serve to regulate the transition between early and late life cycle events, Ser\textsuperscript{326} phosphorylation allowing the release of RNA products that would become available for other processes in later stages of infection such as packaging or cell-to-cell movement.

Although many examples of mutations affecting RNA synthesis were reported previously (48, 51–55), our data suggest the existence of a possible link between phosphorylation of the RdRp at a specific site within the catalytic domain and the regulation of viral replication. The evolutionary conservation of the target residue from alphavirus to picornavirus RdRps (27) suggests that it might constitute a general feature of RNA viruses. Further studies are required to elucidate whether this is indeed the case.

The kinase(s) responsible for modification of TYMV RdRp remain unknown. That phosphorylation occurs in cells from species as distantly related as insects and plants suggests that the kinase is broadly distributed and perhaps highly conserved. Once identified, kinase(s) or phosphatase(s) that can modify RdRp phosphorylation status will constitute attractive targets for the development of anti-viral drugs.

In summary, the key finding reported here is that mutations of specific TYMV RdRp phosphorylation sites critically affect viral replication. Our results suggest that phosphorylation may be involved both in the control of RdRp stability and RdRp function. Such advances not only provide guidance for future biochemical experiments but also provide a framework for future studies on the mechanisms underlying replication and pathology of plus-strand RNA viruses, insight that could prove critical for developing methods to inhibit or contain an infection.

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REFERENCES
1. van Regenmortel, M. H. V., Fauquet, C. M., Bishop, D. H. L., Carstens, E. B., Estes, M. K., Lemon, S. M., Maniloff, J., Mayo, M. A., McGeoch, D. J., Pringle, C. R., and Wickner, R. B. (eds) (2000) Virus Taxonomy: Seventh Report of the International Committee on Taxonomy of Viruses. Academic Press, San Diego, CA.
2. Ahlquist, P., Noueiry, A. O., Lee, W. M., Kushner, D. B., and Dye, B. T. (2003) J. Virol. 77, 8181–8186.
3. Buck, K. W. (1996) Adv. Virus Res. 47, 159–251.
4. Kapoor, M., Zheng, L., Ramachandra, M., Kusukawa, J., Ebner, K. E., and Padmanabhan, R. (1995) J. Biol. Chem. 270, 19100–19106.
5. Héricourt, F., Blanc, S., Redeker, V., and Jupin, I. (2000) Biochem. J. 349, 417–425.
6. Kim, S. H., Palukaitis, P., and Park, Y. I. (2002) EMBO J. 21, 2292–2300.
7. Kim, S. J., Kim, J. H., Kim, Y. G., Lim, H. S., and Oh, J. W. (2004) J. Biol. Chem. 279, 50031–50041.
8. Joyce, C. M., and Steitz, T. A. (1995) J. Virol. 71, 110–114.
9. Ahlquist, P., Noueiry, A. O., Lee, W. M., Kushner, D. B., and Dye, B. T. (2003) J. Virol. 77, 8181–8186.
10. Bressanelli, S., Tomei, L., Roussel, A., Incitti, I., Vitale, R. L., Mathieu, M., De Francesco, R., and Rey, F. A. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 13034–13039.
11. Butcher, S. I., Grimes, J. M., Makeyev, E. V., Bamford, D. H., and Stuart, D. I. (2001) Nature 410, 235–240.
12. Thompson, A. A., and Peersen, O. B. (2004) EMBO J. 23, 3462–3471.
13. Rechsteiner, M., and Rogers, S. W. (1996) Trends Biochem. Sci. 21, 267–271.
14. Dean, A. M., and Koshland, D. E. (1990) Science 249, 1044–1046.
15. Sweeney, H. L., Yang, Z., Zhi, G., Stull, J. T., and Trybus, K. M. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 1490–1494.
16. Waignmann, E., Chen, M. H., Bachmaier, R., Ghoshroy, S., and Citovsky, V. (2000) EMBO J. 19, 4875–4884.
17. Michell, B. J., Harris, M. B., Chen, Z. P., Ju, H., Venema, V. J., Blackstone, M. A., Huang, W., Venema, R. C., and Kemp, B. E. (2002) J. Biol. Chem. 277, 43244–43251.
18. Jia, J., Tong, C., Wang, B., Luo, L., and Jiang, J. (2004) Nature 432, 1045–1050.
19. Bransom, K. L., Weiland, J. I., Tsai, C. H., and Dreher, T. W. (1995) Virolology 206, 403–412.
20. Chattopadhyay, D., and Banerjee, A. K. (1987) Cell 49, 407–414.
21. Chang, T. L., Reiss, C. S., and Huang, A. S. (1994) J. Virol. 68, 4980–4987.
22. Pattnaik, A. K., Hwang, L., Li, T., Englund, N., Marthur, M., Das, T., and Banerjee, A. K. (1997) J. Virol. 71, 8167–8175.
23. De, I., Fata-Hartley, C., Sawicki, S. G., and Sawicki, D. L. (2003) J. Virol. 77, 13106–13116.
24. Evans, M. J., Rice, C. M., and Goff, S. P. (2004) Proc. Natl. Acad. Sci. U. S. A. 101, 13038–13043.
25. Neddermann, P., Quintavalle, M., Di Petro, C., Clementi, A., Cerretani, M., Altamura, S., Bartholomew, L., and De Francesco, R. (2004) J. Virol. 78, 13306–13314.
26. Shapka, N., Stork, J., and Nagy, P. D. (2005) Virology 333, 65–78.
27. Kratz, C., Eckerskorn, C., Janke, C., and Muller, M. A., Huang, W., Venema, R. C., and Kemp, B. E. (2002) J. Virol. 76, 1045–1050.
28. Gohara, D. W., Crotty, S., Arnold, J. J., Yoder, J. D., Andino, R., and De Francesco, R. (2004) J. Virol. 78, 13306–13314.
29. Gohara, D. W., Arnold, J. J., and Cameron, C. E. (2000) J. Biol. Chem. 275, 25523–25532.
30. Gohara, D. W., Arnold, J. J., and Cameron, C. E. (2004) Biochemistry 43, 5149–5158.
31. Arnold, J. L., Vignuzzi, M., Stone, J. K., Andino, R., and Cameron, C. E. (2005) J. Biol. Chem. 280, 25706–25716.
32. Fata, C. L., Sawicki, S. G., and Sawicki, D. L. (2002) J. Virol. 76, 8632–8640.
33. Cheney, I. W., Naim, S., Lai, V. C., Dempsey, S., Bellows, D., Walker, M. P., Shim, J. H., Horscroft, N., Hong, Z., and Zhong, W. (2002) Virology 297, 298–306.