

Phosphorylation of the Potyvirus Capsid Protein by Protein Kinase CK2 and Its Relevance for Virus Infection^W

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We reported previously that the capsid protein (CP) of *Potato virus A* (PVA) is phosphorylated both in virus-infected plants and in vitro. In this study, an enzyme that phosphorylates PVA CP was identified as the protein kinase CK2. The α -catalytic subunit of CK2 (CK2 α) was purified from tobacco and characterized using in-gel kinase assays and liquid chromatography–tandem mass spectrometry. The tobacco CK2 α gene was cloned and expressed in bacterial cells. Specific antibodies were raised against the recombinant enzyme and used to demonstrate the colocalization of PVA CP and CK2 α in infected tobacco protoplasts. A major site of CK2 phosphorylation in PVA CP was identified by a combination of mass spectrometric analysis, radioactive phosphopeptide sequencing, and mutagenesis as Thr-242 within a CK2 consensus sequence. Amino acid substitutions that affect the CK2 consensus sequence in CP were introduced into a full-length infectious cDNA clone of PVA tagged with green fluorescent protein. Analysis of the mutant viruses showed that they were defective in cell-to-cell and long-distance movement. Using in vitro assays, we demonstrated that CK2 phosphorylation inhibited the binding of PVA CP to RNA, suggesting a molecular mechanism of CK2 action. These results suggest that the phosphorylation of PVA CP by CK2 plays an important regulatory role in virus infection.

INTRODUCTION

A rapidly growing body of evidence suggests that in several animal viruses, virion assembly and dissociation are regulated by protein phosphorylation. For example, phosphorylation of the major structural proteins of *Herpes simplex virus* type 1 triggers tegument dissociation (Morrison et al., 1998). In the case of *Human hepatitis B virus*, the core protein phosphorylation regulates pre-genomic RNA encapsidation (Gazina et al., 2000). A defect in polyomavirus assembly was associated with a mutation at the phosphorylation site of the major capsid protein (CP) VP1 (Li and Garcea, 1994). In yet another study, phosphorylation of the Cap24 structural protein of *Human immunodeficiency virus* type 1 was shown to be necessary just after the entry of the virus into the target cell, suggesting that phosphorylation promotes the disassembly of the viral capsid (Cartier et al., 1999). Recently, new evidence has appeared indicating that phosphorylation of *Rubella virus* capsid may regulate virion assembly by changing the affinity of CP for RNA (Law et al., 2003). In contrast to animal viruses, there is little information regarding the role of CP phosphorylation in the infection cycle of plant viruses. It has been reported that the CP of the *Cauliflower mosaic virus* (CaMV)

is phosphorylated in vitro by a virion-associated protein kinase (Martinez-Izquiero and Hohn, 1987). In a follow-up study, point mutations affecting the phosphorylation sites of CaMV CP were shown to reduce virus accumulation levels significantly (Leclerc et al., 1999). Another study has demonstrated that in vitro phosphorylation of *Potato virus X* triggers cotranslational virion disassembly (Atabekov et al., 2001).

In a previous study (Ivanov et al., 2001), we reported that the CP of *Potato virus A* (PVA), a potyvirus, is phosphorylated by a plant protein kinase activity in vivo and in vitro. Furthermore, Ser/Thr-specific phosphorylation of PVA CP reduced its affinity for RNA, suggesting a mechanism to regulate the formation and/or stability of viral ribonucleoprotein complexes. In support of our findings, a recent report described Ser/Thr phosphorylation of the CP of another potyvirus, *Plum pox virus* (PPV) (Fernández-Fernández et al., 2002). That study also showed that PPV CP is modified by O-linked N-acetylglucosamine, a modification reciprocal to phosphorylation in many eukaryotic proteins (Comer and Hart, 2000).

In addition to virion packaging, the CPs of potyviruses are involved in the movement of infectious viral RNA from cell to cell and over long distances (Dolja et al., 1994). In many other plant viruses, including the most thoroughly studied, *Tobacco mosaic virus* (TMV), these functions are executed by virus-encoded nonstructural movement proteins (MPs). The phosphorylation of TMV MP has been demonstrated in vitro, in the baculovirus–insect cell system, in infected protoplasts, in infected plants, and in MP-expressing transgenic plants (Atkins et al., 1991; Watanabe et al., 1992; Citovsky et al., 1993; Waimann et al., 2000). Phosphorylation can affect several MP functions (Karpova

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et al., 1999; Kawakami et al., 1999; Waigmann et al., 2000), and a possible role of MP phosphorylation in the regulation of inter-cellular macromolecular trafficking has been discussed (Lee and Lucas, 2001). However, there is still limited information regarding the identities of the protein kinases that phosphorylate MPs. A plant protein kinase with the biochemical properties of CK2 (formerly casein kinase II) has been shown to interact specifically with the MP of *Tomato mosaic virus*, a virus closely related to TMV, and to phosphorylate it in vitro (Matsushita et al., 2000). This result was confirmed recently using the recombinant α -catalytic subunit of CK2 from tobacco (Matsushita et al., 2003).

Our previous studies have established that PVA CP and TMV MP are both substrates for the same Ser/Thr kinase activity from tobacco leaves (Ivanov et al., 2001). Here, we identified tobacco CK2 as the enzyme responsible for PVA CP phosphorylation both in vivo and in vitro and mapped Thr-242 as the main phosphorylation site within a triple CK2 consensus sequence in the C terminus of the protein. Point mutations that affect the CK2 consensus sequence in PVA CP were introduced into the full-length infectious cDNA clone of PVA tagged with green fluorescent protein (GFP), and the resulting mutants were found to have a movement-deficient phenotype. The effect of CK2 phosphorylation on the RNA binding function of PVA CP was studied, and the affinity of the protein for RNA was shown to decrease upon CK2 phosphorylation. Based on these results, we discuss the role of phosphorylation/dephosphorylation events mediated by CK2 and other yet unidentified protein kinases and phosphatases in the viral infection cycle.

RESULTS

PVA CP Is Phosphorylated by CK2 in Vitro

PVA CP was expressed in bacteria as a fusion protein with an N-terminal hexahistidine affinity tag and was purified to homogeneity by immobilized metal affinity chromatography. We showed previously that PVA CP obtained using this approach is folded similarly to the protein synthesized in planta, because they are phosphorylated at the same sites (Ivanov et al., 2001). To examine the possible role of CK2 in the phosphorylation of PVA CP, we performed in vitro kinase assays with bacterially expressed PVA CP and tobacco protein extracts. Because CK2 is unique among protein kinases in its ability to use both ATP and GTP as phosphoryl group donors (Allende and Allende, 1995; Niefind et al., 1999), the phosphorylation of PVA CP was tested in the presence of each of the two nucleotide triphosphates. Another biochemical property of CK2 that makes it different from other kinases is its high sensitivity to inhibition by heparin (Hathaway et al., 1980). Therefore, the effect of heparin on the phosphorylation of PVA CP also was examined. As shown in Figure 1A, autoradiography revealed that PVA CP was phosphorylated in the presence of both GTP and ATP and that this reaction was inhibited by heparin. These data suggested that PVA CP phosphorylation could be attributed to a CK2-like enzyme. We also observed that GTP was a better phosphoryl donor than ATP in the presence of Mn^{2+} (Figures 1A and 1B), in agreement with the published kinetic parameters of CK2 (Gatica et al., 1993).

Next, we performed in vitro kinase reactions using the isolated α -catalytic subunit of CK2 and the recombinant PVA CP

and TMV MP as substrates. We used the well-studied α -catalytic subunit of CK2 from maize (rmCK2 α ; Boldyreff et al., 1993), which is >90% identical at the protein level to its homolog from tobacco (Salinas et al., 2001). PVA genome-linked protein (VPg) was included in the assays to verify our previous results indicating that PVA VPg is a substrate for a protein kinase other than PVA CP and TMV MP (Ivanov et al., 2001). Indeed, the results presented in Figure 1B show that PVA CP and TMV MP were phosphorylated readily by rmCK2 α in the presence of ATP and GTP, whereas only a minor phosphorylation of PVA VPg by CK2 was detected. In agreement with our earlier data on the cation specificity of the plant protein kinase activity that phosphorylates PVA CP and TMV MP (Ivanov et al., 2001), rmCK2 α exhibited the same preference for Mn^{2+} over Mg^{2+} and was not stimulated by Ca^{2+} (data not shown).

The in Vivo Phosphorylation of PVA CP Is Inhibited by 5,6-Dichloro-1-(β -D-Ribofuranosyl)Benzimidazole, a Cell-Permeable Inhibitor of CK2

To test the involvement of CK2 in the phosphorylation of PVA CP in vivo, leaf discs from PVA-infected tobacco plants were vacuum-infiltrated with a buffer containing ^{33}P -orthophosphate, 0.5% (v/v) DMSO, and a specific CK2 inhibitor, 5,6-dichloro-1-(β -D-ribofuranosyl)benzimidazole (DRB). The inhibitor was added at a concentration of 500 μ M, which was reported to be effective in tobacco (Hidalgo et al., 2001). Unlike heparin, DRB is a cell-permeable compound; therefore, it can be used as an inhibitor of CK2 in vivo. A control experiment was performed to verify that the addition of DMSO did not inhibit PVA CP phosphorylation. In this experiment, PVA-infected leaf discs were infiltrated with the ^{33}P -orthophosphate solution containing 0.5% (v/v) DMSO without the addition of DRB. In another control experiment, the same solution was vacuum-infiltrated into the mock-infected leaf discs to verify the virus-specific nature of phosphorylation. The leaf discs were incubated overnight to allow metabolic incorporation of the radioactive phosphate into PVA CP, the cells were lysed, and the protein was immunoprecipitated with polyclonal anti-PVA antibodies. Figure 1C shows that the ^{33}P -labeled protein was immunoprecipitated only from PVA-infected plant material. Furthermore, the protein was recognized specifically by the mouse monoclonal anti-PVA antibody, confirming its identity as PVA CP. The addition of DRB, the cell-permeable inhibitor of CK2, markedly reduced PVA CP phosphorylation in virus-infected cells (Figure 1C, lane 3). This result strongly suggested that PVA CP was phosphorylated by CK2 during the virus infection cycle. A control infiltration without DRB showed that 0.5% DMSO did not affect phosphorylation.

Identification of Tobacco CK2 as the Kinase Involved in PVA CP Phosphorylation

To further characterize the enzymatic activity involved in the phosphorylation of PVA CP in tobacco, we performed affinity purification of the kinase. Heparin-Sepharose was chosen as an affinity matrix for purification, because it is known to strongly retain plant CK2 even in the presence of moderate salt concentrations (Klimczak et al., 1992). As shown in Figure 2 (top), PVA CP

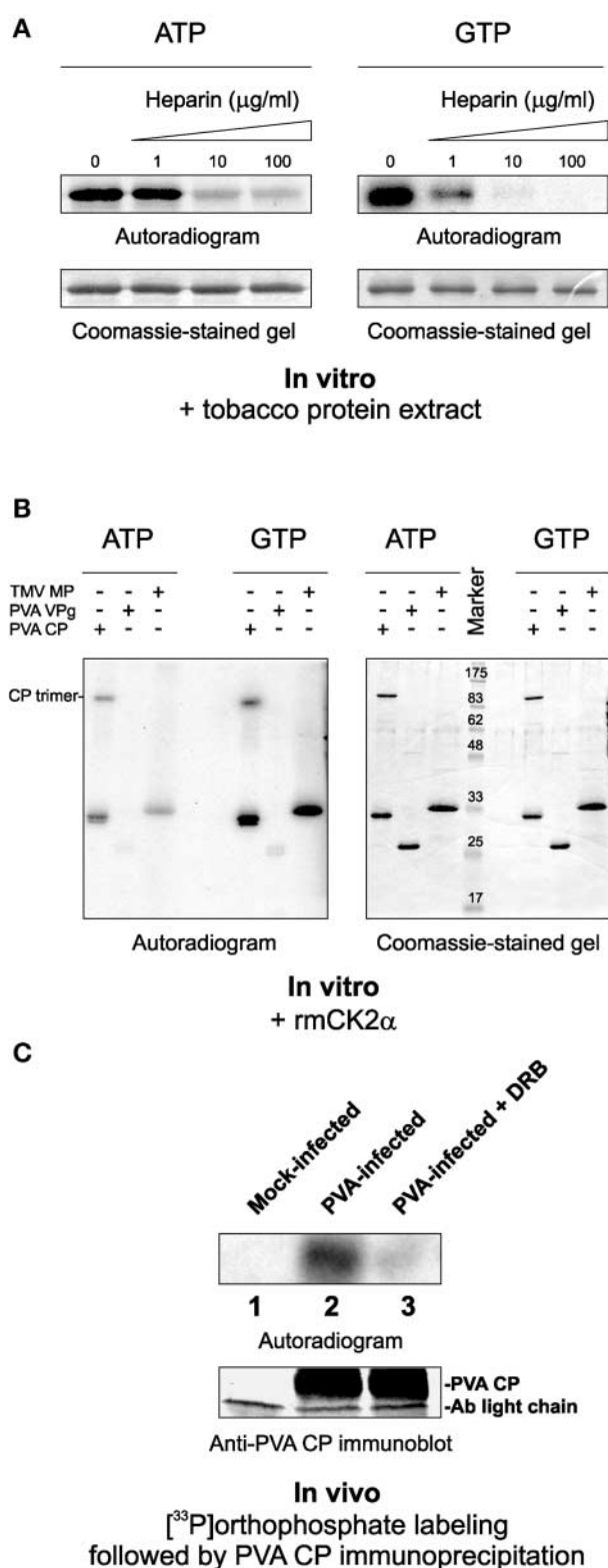


Figure 1. Evidence for the Involvement of CK2 in the Phosphorylation of PVA CP in Vitro and in Vivo.

(A) Effect of increasing amounts of heparin on the phosphorylation of PVA CP in vitro.

kinase activity was eluted from the column as a major peak between 580 and 700 mM KCl. The isolated kinase was able to use GTP as a phosphoryl donor, demonstrating the enzymatic properties characteristic of CK2. To further characterize the enzyme eluted from heparin-Sepharose, we examined whether it has the same substrate specificity as rmCK2 α . In complete agreement with the results described above for the maize CK2, the kinase efficiently phosphorylated PVA CP and TMV MP, whereas virtually no phosphorylation of PVA VPg was observed (Figure 2, bottom left).

As a next step, we used an in-gel kinase assay to determine the size of the enzyme and its sensitivity to inhibition by heparin. The column fractions containing the highest kinase activity were subjected to electrophoresis on an SDS-polyacrylamide gel containing PVA CP incorporated into the gel matrix. After electrophoresis, resolved proteins were allowed to renature and then were assayed for protein kinase activity in the presence of γ - ^{32}P -GTP. As shown in Figure 2 (bottom right), the in-gel kinase assay revealed one major radioactive band with an apparent molecular mass of 39 kD. By contrast, no radiolabeled band was detected when the gel was incubated in the presence of γ - ^{32}P -GTP plus 20 $\mu\text{g/ml}$ heparin or when PVA CP was omitted from the gel matrix. This result allowed us to conclude that the kinase activity purified on heparin-Sepharose was able to phosphorylate PVA CP in a heparin-sensitive manner using GTP as a phosphoryl donor. Moreover, the molecular mass of the identified kinase (39 kD) was identical to that of the recently reported α -catalytic subunit of CK2 from tobacco (tCK2 α ; Salinas et al., 2001). Ultimately, liquid chromatography–tandem mass spectrometry (LC-MS/MS) was used to prove the identity of the 39-kD protein kinase. The protein band was excised from an SDS-polyacrylamide gel, digested with trypsin, and analyzed by LC-MS/MS. Two unique peptide sequence tags matching the α -catalytic subunit of tobacco CK2 were identified (Figure 2, table). The probability of false identification using the peptide mass data together with the sequences of 7 and 10 amino acids was calculated to be $<10^{-16}$ (for calculation method, see Mann and Wilm, 1994). In addition to tCK2 α , LC-MS/MS analysis revealed the presence of three other proteins. However, none of them was a protein kinase. Thus, we identified tobacco CK2 as the kinase responsible for the phosphorylation of PVA CP.

Expression, Purification, and Catalytic Activity of the Recombinant tCK2 α

To produce recombinant tCK2 α in bacterial cells, the full-length gene of tCK2 α (Salinas et al., 2001) was amplified from a tobacco

(B) PVA CP and TMV MP, but not PVA VPg, are phosphorylated efficiently by the α -catalytic subunit of recombinant maize CK2.

(C) Effect of DRB, a cell-permeable inhibitor of CK2, on the phosphorylation of PVA CP in vivo. Leaf discs from mock- or PVA-infected tobacco plants were labeled metabolically using ^{33}P -orthophosphate in the presence or absence of 500 μM DRB. PVA CP was immunoprecipitated and examined by protein gel blot analysis followed by autoradiography. The positions of PVA CP and a closely migrating antibody (Ab) light chain are indicated at right.

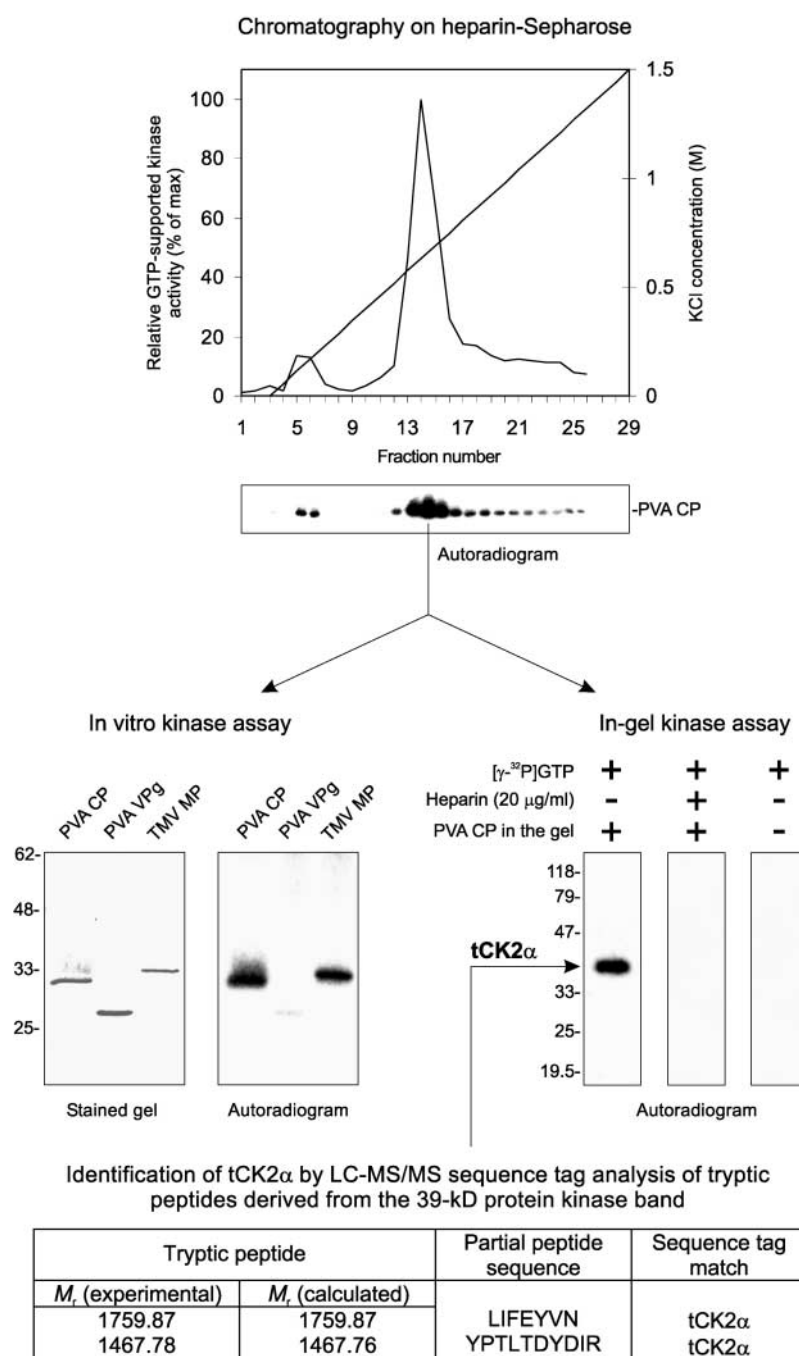


Figure 2. An In-Gel Kinase Assay in Conjunction with Peptide Sequence Tag Analysis Detects Tobacco CK2 as the Kinase Involved in PVA CP Phosphorylation.

The top panel shows the elution profile of kinase activity from heparin-Sepharose as determined by kinase assays of collected fractions. Fraction 14, which contained maximal protein kinase activity, was assayed further for the phosphorylation of PVA CP, PVA VPg, and TMV MP in the presence of γ - ^{32} P-GTP (bottom left). Alternatively, the proteins in fraction 14 were precipitated with trichloroacetic acid, solubilized, and subjected to electrophoresis on a 12% SDS-polyacrylamide gel that was polymerized in the presence of 0.1 mg/mL PVA CP. The in-gel kinase assay was performed as described in Methods, and the ^{32} P-labeled proteins were visualized by autoradiography (bottom right). Control kinase assays were performed in the presence of 20 μ g/mL heparin or on a gel polymerized without PVA CP. The catalytically active band was excised from a 12% SDS-polyacrylamide gel, digested with trypsin, and subjected to LC-MS/MS sequence tag analysis. The position of tobacco CK2 α is indicated with an arrow. The migration of molecular mass standards (in kilodaltons) is shown at left on each gel. The table at the bottom of the figure summarizes the results of LC-MS/MS sequence tag analysis of tryptic peptides derived from the 39-kD protein kinase band.

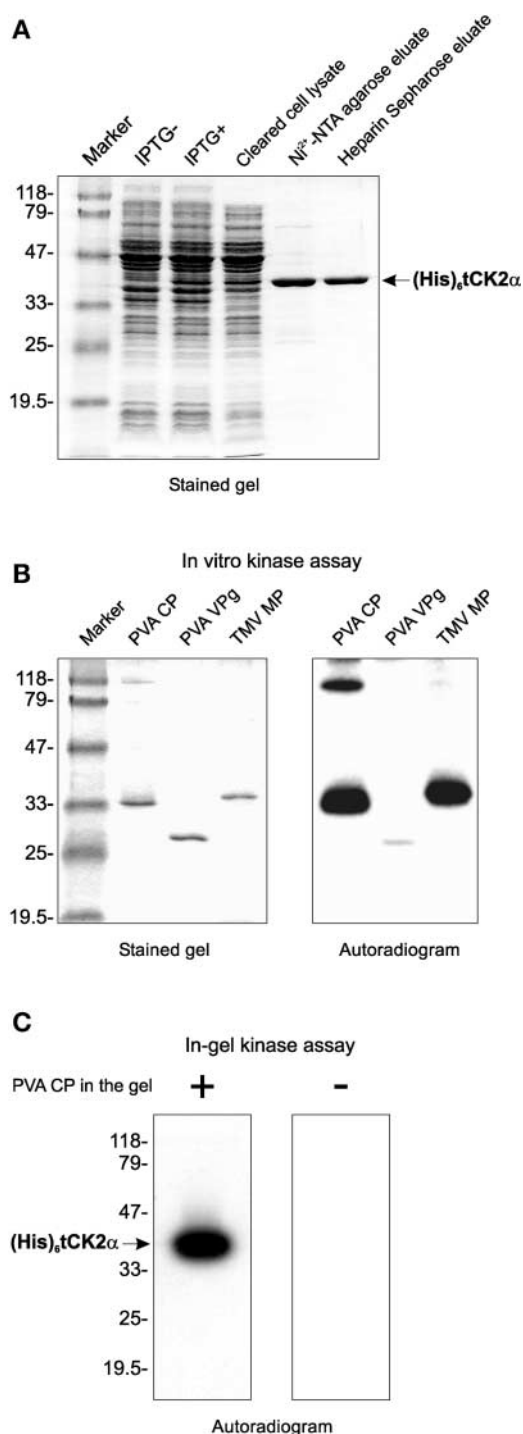


Figure 3. Purification and Catalytic Activity of the Recombinant (His)₆tCK2α Produced in *E. coli* Cells.

(A) Electrophoretic patterns of bacterial cell lysates before (–) and after (+) isopropylthio-β-galactoside (IPTG) induction cleared cell lysate and column eluates from Ni²⁺-NTA agarose and heparin-Sepharose.

(B) Purified recombinant (His)₆tCK2α was assayed for the phosphorylation of PVA CP, TMV MP, and PVA VPg in the presence of γ-³²P-GTP.

cDNA library and cloned in frame with a hexahistidine affinity tag into the pQE-30 expression vector. The *Escherichia coli* strain M15[pREP4] harboring the constructed plasmid produced a detectable amount of soluble (His)₆tCK2α. The enzyme was purified to homogeneity by sequential chromatography on nickel-nitrilotriacetic acid (Ni²⁺-NTA) agarose and heparin-Sepharose (Figure 3A). The ability of the isolated tCK2α to phosphorylate PVA CP and TMV MP was tested in the presence of γ-³²P-GTP. Both substrate proteins were phosphorylated efficiently, whereas the control reaction with PVA VPg gave a very low yield of labeled product (Figure 3B). To verify that the observed phosphorylation of PVA CP was attributable to the activity of CK2, we performed an in-gel kinase assay in the presence of γ-³²P-GTP. The PVA CP kinase migrated at ~39 kD, confirming its identity as tCK2α, and no radioactive band was detected when PVA CP was omitted from the gel matrix (Figure 3C). The catalytically active recombinant tCK2α was used to raise polyclonal antiserum in rabbit. Anti-tCK2α antibodies were affinity-purified as described by Peranen (1992), and their specificity was confirmed by immunoblot analysis (Figure 4A). These antibodies then were used for immunofluorescent detection of tCK2α in tobacco protoplasts. Preimmune serum sample was processed similarly and used as a negative control.

Colocalization of PVA CP and tCK2α in Infected Protoplasts

To determine whether PVA CP and tCK2α colocalize in infected protoplasts, we performed double-label indirect immunofluorescence experiments with anti-PVA CP and anti-tCK2α antibodies. Representative images obtained by confocal microscopy are shown in Figure 4B. In agreement with the literature data (Faust and Montenarh, 2000), tCK2α was found in the nucleus and the cytoplasm, whereas PVA CP was detected in cytoplasmic patches that often were located near the nucleus. Digital superimposition of the colorized fluorescence images revealed colocalization of PVA CP and tCK2α (Figure 4B). The observed colocalization of the two proteins further supported the notion that PVA CP is the *in vivo* substrate for CK2. No specific fluorescence was detected when cells were incubated with secondary antibodies alone or with preimmune serum, and no green signal was observed for the noninfected protoplasts (data not shown).

Thr-242 Is the Major Site of CK2 Phosphorylation in PVA CP

Sequences in PVA CP containing the CK2 phosphorylation site motif (S/T)XX(D/E) (reviewed by Meggio and Pinna, 2003) were identified using the ScanProsite program tool at the Expasy molecular biology server of the Swiss Institute of Bioinformatics (<http://www.expasy.ch>). A particularly strong consensus sequence (242-TTSEED-247) containing two Thr and one Ser res-

(C) In-gel kinase assay of recombinant (His)₆tCK2α using PVA CP as a substrate. The position of (His)₆tCK2α is indicated with an arrow. The migration of molecular mass standards (in kilodaltons) is shown at left on each gel. All substrate proteins are His fusions.

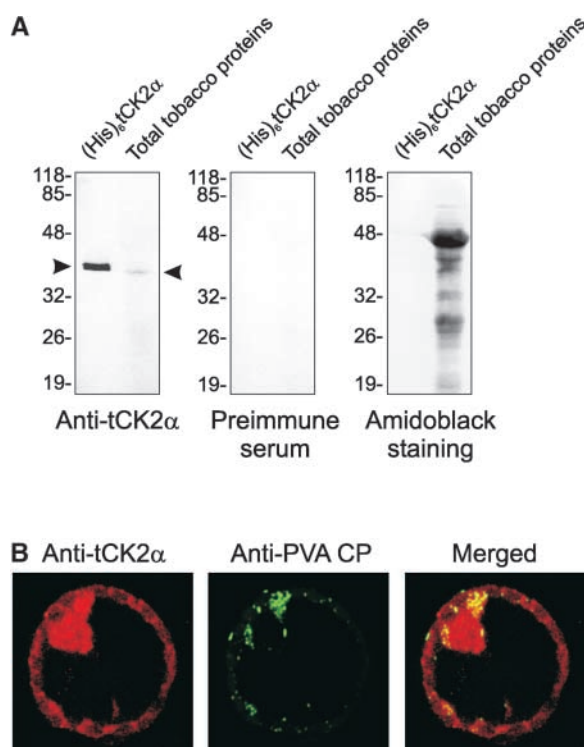


Figure 4. Double-Label Immunofluorescence Confocal Microscopy Reveals Colocalization of tCK2 α and PVA CP in Virus-Infected Tobacco Protoplasts.

(A) Validation of anti-tCK2 α antibody specificity by immunoblot analysis. Samples containing purified (His) $_6$ tCK2 α (8 ng) or total protein extract from tobacco leaves were subjected to SDS-PAGE, transferred to a membrane, and probed with affinity-purified anti-tCK2 α antibody or pre-immune serum. The positions of (His) $_6$ tCK2 α and tCK2 α are marked with arrowheads. The migration of molecular mass standards (in kilodaltons) is indicated at left on each blot. The gel at right shows amido black staining. **(B)** Infected protoplasts were fixed and processed for double-label indirect immunofluorescence using antibodies that detect tCK2 α (red signal) and PVA CP (green signal). Colocalization of the two signals is shown in the merged image as yellow. The confocal images were collected with a focal depth of 0.54 μ m. Shown are projections of two optical sections.

idues available for CK2 phosphorylation was identified in the tryptic peptide Asn-229 to Arg-250. To begin the experimental work to characterize the sites of protein modification, PVA CP was phosphorylated in a reconstituted system containing tobacco protein extract and γ - ^{33} P-ATP and digested with trypsin, and the resulting peptides were resolved by reverse-phase HPLC. As shown in Figure 5A, radioactivity was found mainly in one peak, although several smaller radioactive peaks also were present. Matrix-assisted laser desorption/ionization–time of flight (MALDI-TOF) mass spectrometric analysis was performed to characterize the peptide content of the main radioactive peak. Mass spectrometry revealed the presence of a single peptide of M_r 2430.7, which was identified as 229-NSNTNMFGLDGN-VTTSEEDTER-250 (Asn-229 to Arg-250) with a calculated mass of 2430 kD (Figure 5A, inset).

As indicated above, the same peptide was found previously to contain the CK2 consensus sequence 242-TTSEED-247. However, further mass spectrometric analysis was hampered by poor ionization of the phosphorylated form of this peptide. Therefore, we used another experimental approach based on peptide mapping and radioactive phosphate-release sequencing. PVA CP was extracted by LiCl from virus particles and phosphorylated in a reconstituted system using isolated rmCK2 α or tobacco protein extract as a kinase source. Previously, we demonstrated that PVA CP is phosphorylated in vitro by tobacco protein kinase activity on multiple sites (Ivanov et al., 2001). Consistent with this finding, we again observed at least five radioactive spots on the tryptic phosphopeptide map of PVA CP after its incubation with tobacco protein extract (Figure 5B, top left). By contrast, a single phosphopeptide spot was detected on the map of PVA CP phosphorylated by isolated rmCK2 α (Figure 5B, bottom left). This spot had exactly the same mobility as one of the spots on the first map, indicating that both correspond to the same phosphopeptide.

To determine at which position in this peptide a phosphorylated residue exists, the labeled peptide material was recovered from each cellulose plate and subjected to radioactive phosphate-release sequencing. Sequencing confirmed that the phosphopeptides extracted from two overlapping spots were identical. Most of the radioactivity was released at Edman degradation cycle 14, suggesting a primary phosphorylation site (Figure 5B, inset box, top). In a separate experiment, we examined the phosphoamino acid content of the sequenced phosphopeptide. Two-dimensional phosphoamino acid analysis revealed that the peptide contained only phosphothreonine (Figure 5B, inset box, bottom). Together, these results indicated that the studied peptide should contain a Thr residue at position 14. The only tryptic peptide matching this criterion was again identified as 229-NSNTNMFGLDGNVTTSEEDTER-250 (Asn-229 to Arg-250). Thus, we determined that Thr-242, the first Thr residue in the triple CK2 consensus sequence 242-TTSEED-247, is the major site of PVA CP phosphorylation by CK2.

To further prove that Thr-242 within the core domain of the protein (Figure 6A) is phosphorylated by CK2, we purified a bacterially expressed mutant of PVA CP containing a single amino acid substitution of Thr-242 for Asp (T242D) and tested its phosphorylation by isolated CK2. As shown in Figure 6B, the phosphorylation of T242D by rmCK2 α was reduced severalfold compared with that of the wild-type protein. The presence of small but detectable levels of radioactivity in the mutant protein indicated that in the absence of Thr-242, the kinase phosphorylated the downstream residues in the triple CK2 consensus sequence less efficiently. When Thr-243 was converted to Asp (T243D), the mutation had no effect on phosphorylation (Figure 6B, lane T243D), confirming that Thr-242 is a preferred substrate for CK2. Thus, the results described above indicate that within the C-terminal part of PVA CP, Thr-242 is the major single site for CK2.

Effect of Point Mutations That Affect the CK2 Consensus Sequence on Virus Infection

To examine the role of PVA CP phosphorylation by CK2 in viral infection, we constructed a full-length infectious cDNA clone of

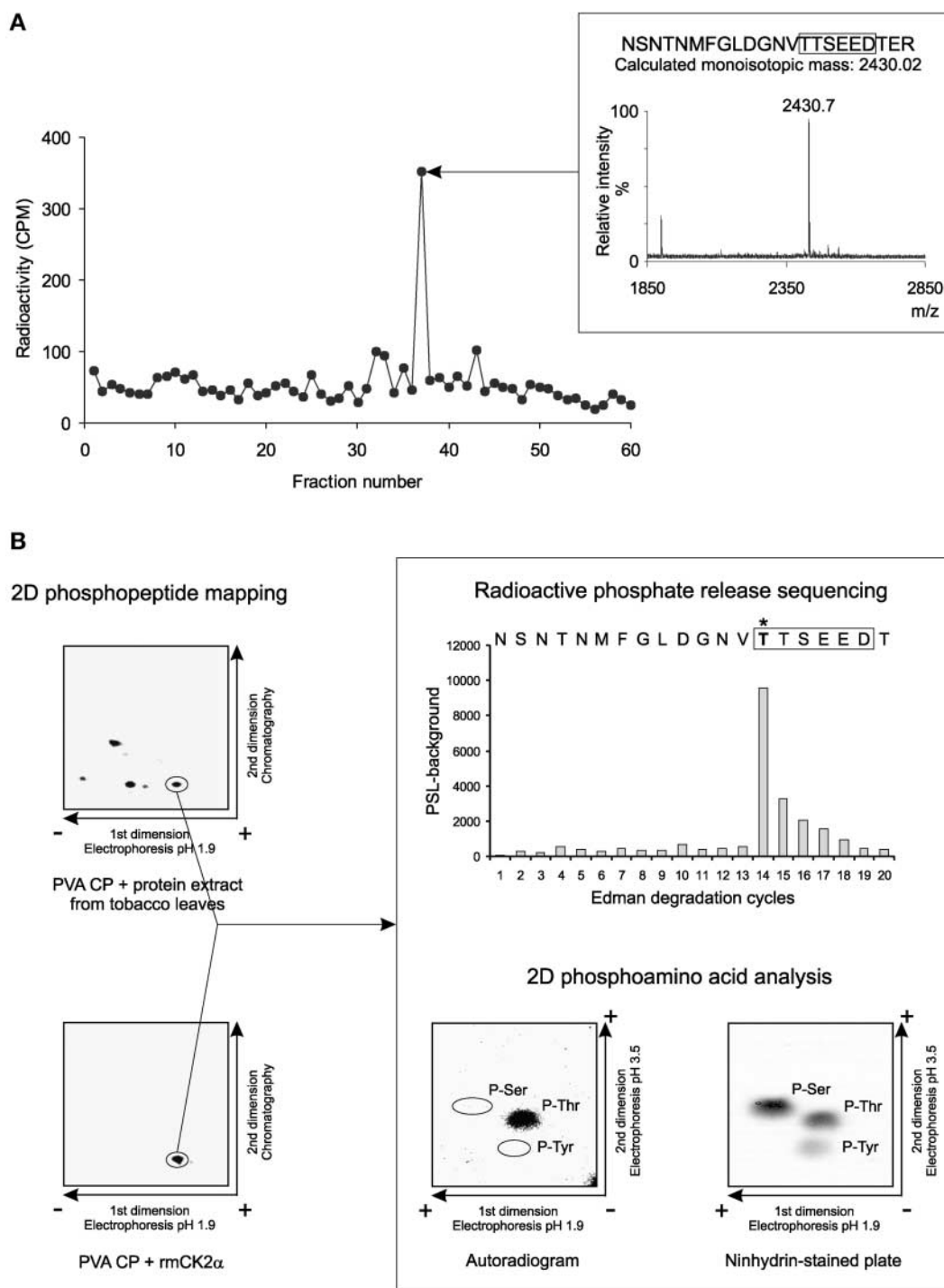


Figure 5. Mapping of the CK2 Phosphorylation Site in PVA CP.

(A) Bacterially expressed His-tagged PVA CP was phosphorylated in vitro and digested with trypsin, and the resulting phosphopeptides were resolved by reverse-phase HPLC. Shown is the distribution of ^{33}P radioactivity in fractions collected from a chromatographic separation. The fraction with the highest label content was analyzed by MALDI-TOF mass spectrometry (inset box). The identified tryptic peptide containing a CK2 consensus sequence (boxed) is shown above the mass spectrum. m/z, mass-to-charge ratio.

(B) Thr-242 is the major CK2 phosphorylation site in PVA CP. Shown are peptide maps of PVA CP phosphorylated by protein extract from tobacco leaves (top left) or rmCK2 α (bottom left). Trypsin-digested peptides were separated by thin layer electrophoresis in the first dimension and chromatography in the second. A phosphopeptide found on both maps was recovered from cellulose plates and subjected to phosphate-release sequencing (inset box, top) and phosphoamino acid analysis (inset box, bottom). Radioactivity released by each cycle of Edman degradation was quantified by phosphoimaging after subtraction of the background. The identified amino acids are shown in one-letter code above the graph. The phosphorylated Thr residue corresponding to Thr-242 is shown in boldface and is marked with an asterisk. The phosphoamino acid composition of the peptide was determined by thin layer two-dimensional electrophoresis followed by autoradiography. The circled regions on the autoradiogram (inset box, bottom left) indicate the positions where phosphoamino acid markers migrated, as determined by ninhydrin staining (inset box, bottom right).

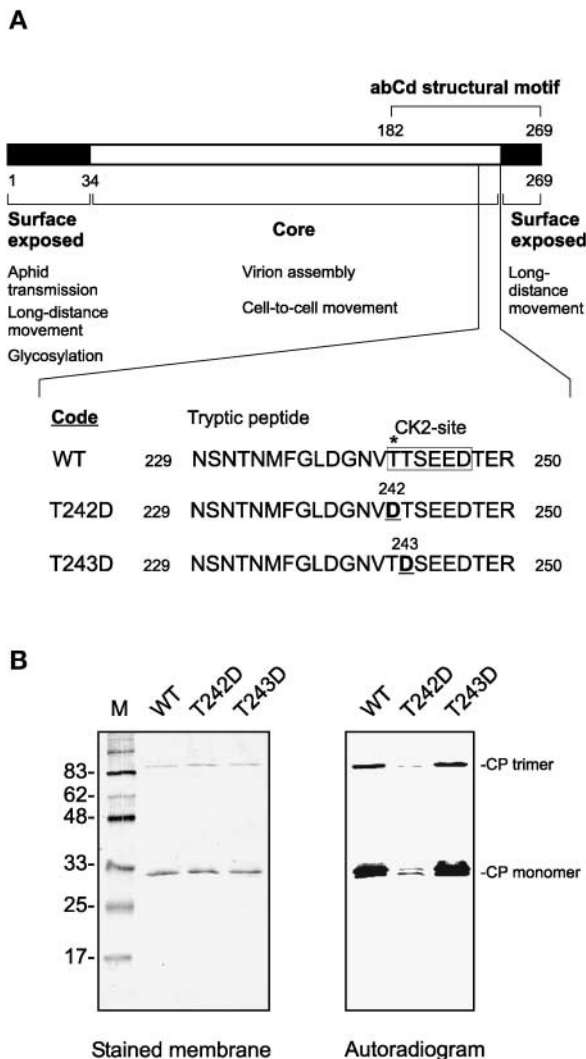


Figure 6. In Vitro Phosphorylation of PVA CP Mutants by the Purified α -Catalytic Subunit of Recombinant Maize CK2.

(A) Scheme of PVA CP and its substitution mutants. The surface-exposed N- and C-terminal domains of the protein (black boxes) are shown together with a predicted abCd structural motif (Baratova et al., 2001) commonly found in RNA binding proteins. The known functions attributed to different regions of the protein (Dolja et al., 1995; Mahajan et al., 1996; Fernández-Fernández et al., 2002) are given below the scheme. The expanded diagram shows the positions of mutagenized Thr-242 and Thr-243 in the CK2-phosphorylated tryptic peptide. The positions of Thr-242 (marked with an asterisk) and the CK2 consensus motif (boxed) are shown in the sequence of wild-type (WT) peptide. Asp residues substituting for Thr residues in mutants T242D and T243D are shown in boldface type and underlined.

(B) Bacterially expressed His-tagged wild-type PVA CP (WT) and its substitution mutants T242D and T243D were assayed for phosphorylation by rmCK2 α . Proteins were separated by 12% SDS-PAGE and stained with Coomassie blue (left gel), and phosphorylation was detected by autoradiography (right gel). The migration of molecular mass standards (lane M) is indicated in kilodaltons at left. Double bands correspond to different PVA CP isoforms.

PVA tagged with GFP (35S-PVA-GFP^{Nlb/CP}). The reporter gene was inserted at the junction of the Nlb and CP genes and flanked by two Nla protease cleavage sites so that GFP could be cleaved out of the polyprotein during processing (Figure 7). Particle bombardment was used to introduce the GFP-tagged virus into leaf epidermal cells of *Nicotiana benthamiana*. Starting from 5 days after inoculation, strong GFP fluorescence was detected in the bombarded leaves, confirming that 35S-PVA-GFP^{Nlb/CP} was fully infectious. At 7 days after inoculation, fluorescence continued to spread from cell to cell in the bombarded leaves and also was detected in the veins of the upper leaves (Figures 7A and 7B).

Several amino acid exchange mutations were introduced into 35S-PVA-GFP^{Nlb/CP} by site-directed mutagenesis. The results described above indicate that Thr-242, when mutated, could be substituted as the site of CK2 phosphorylation by the downstream residues in the triple CK2 consensus sequence. Therefore, to produce phosphorylation-deficient mutants, we had to alter all three potential CK2 phosphorylation sites in PVA CP. Three consecutive amino acids (Thr-242, Thr-243, and Ser-244) (Figure 7) were substituted either by nonphosphorylatable Ala residues or by Asp or Tyr residues mimicking the electrostatic and steric effects of phosphorylation (Dean and Koshland, 1990). The resulting mutant viruses were completely unable to spread both cell to cell and systemically. At 7 days after inoculation, all of the mutants showed the same defective phenotype, with GFP fluorescence restricted to single cells (Figures 7C to 7E). At 20 days after inoculation with the Ala mutant, weak GFP fluorescence appeared in the upper leaves. Reverse transcriptase-mediated (RT) PCR followed by nucleotide sequencing revealed a reverse mutation from Ala-243 to Thr-243, indicating that the virus had to restore at least one CK2 site to remain viable. This finding also proved that the mutant was able to replicate in individual cells.

As a next step, we wanted to verify that the defective phenotypes were not just caused by the amino acid substitutions per se but by altered CK2 phosphorylation. A mutant virus was constructed in which Thr-242, Thr-243, and Ser-244 were left intact but the downstream cluster of acidic residues was replaced with basic Arg residues. In this mutant, Thr-242 could not be phosphorylated because of the disruption of the acidic context within the CK2 consensus sequence. Once again, the mutant virus showed the same defective phenotype. Starting from 7 days after inoculation, GFP fluorescence was detected only in single bombarded cells (Figure 7F). Together, these results demonstrate that the interference with CK2 phosphorylation of PVA CP severely impairs virus propagation in host plants.

Structural Comparison of Wild-Type PVA CP and Its CK2 Phosphorylation-Deficient Mutant

To exclude the possibility that the point mutations exerted their effect through alteration of the CP conformation, we performed a structural comparison of wild-type PVA CP and its CK2 phosphorylation-deficient mutant. For this purpose, the CP mutant having Thr-242, Thr-243, and Ser-244 substituted by nonphosphorylatable Ala residues was expressed in bacteria as a His-tagged fusion protein and purified to homogeneity by immobilized metal affinity chromatography. The overall secondary structure of this mutant was compared with that of the wild-type PVA CP us-

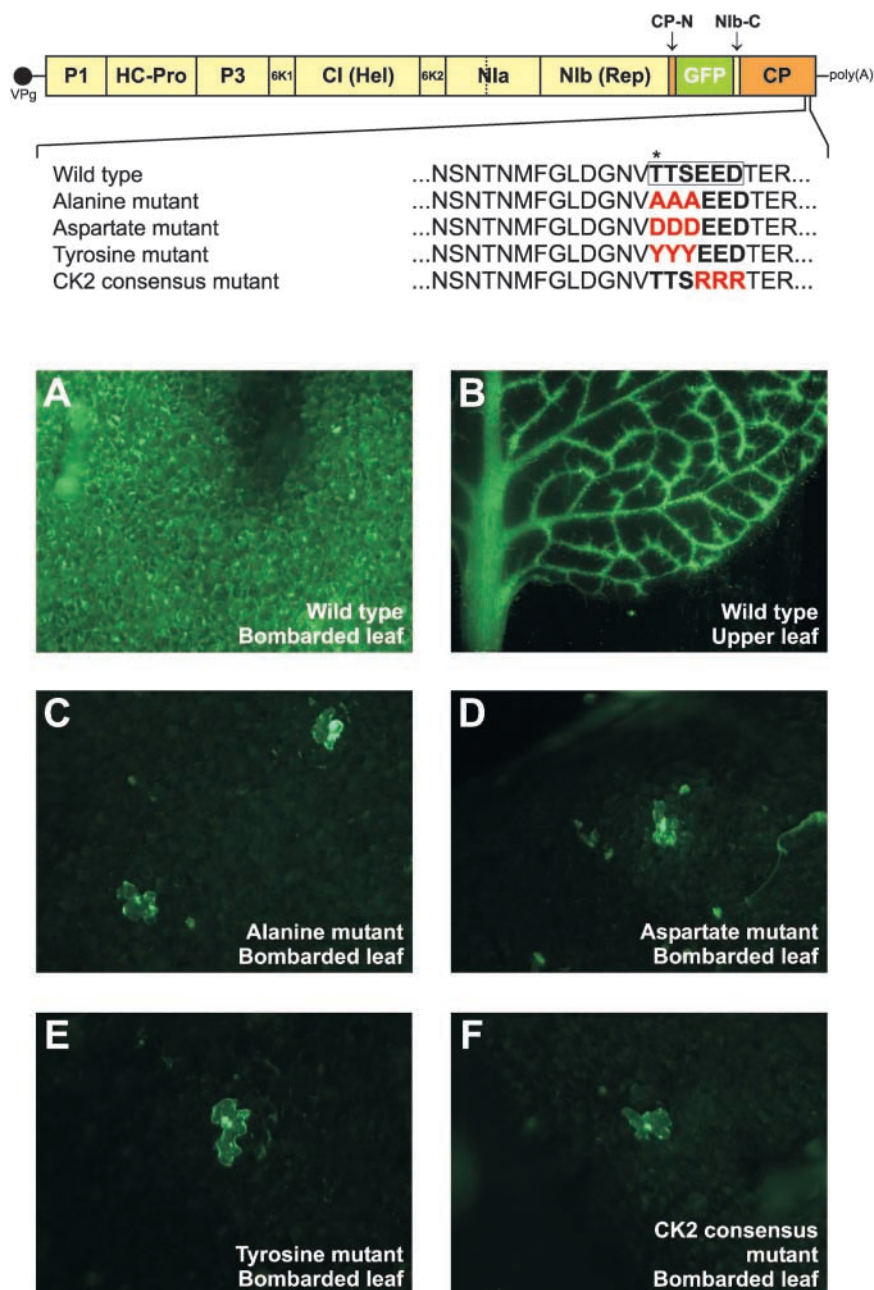


Figure 7. GFP Fluorescence in Leaves of *N. benthamiana* at 7 Days after Inoculation with Wild-Type 35S-PVA-GFP^{Nlb/CP} and Various Mutants.

(A) and (B) Wild-type 35S-PVA-GFP^{Nlb/CP}.

(C) Phosphorylation-deficient mutant.

(D) and (E) Phosphorylation-mimicking mutants.

(F) CK2 consensus mutant.

At top is a scheme of the GFP-tagged virus and its substitution mutants. The positions of Thr-242 (marked with an asterisk) and the CK2 consensus motif (boxed, in boldface type) are shown in the sequence of wild-type PVA CP. Amino acid substitutions are marked in red.

ing circular dichroism (CD) spectroscopy. As shown in Figure 8A, the CD spectra of wild-type PVA CP and its mutant were almost identical, indicating that the proteins have equivalent conformations. Thus, we concluded that the CK2 phosphorylation-negative mutations did not induce conformational changes in the CP.

To further prove this notion, we studied whether the CK2 phosphorylation-negative mutations have any effect on the self-polymerization of PVA CP in bacterial cells. As shown in Figure 8B, analysis by electron microscopy revealed no major difference in the morphology of the virus-like particles formed in bacterial

cells expressing the wild-type PVA CP and its CK2 phosphorylation-deficient mutant. This result demonstrated that the CK2 phosphorylation-negative mutations did not disturb the inter-subunit interactions between the CP monomers required for self-polymerization, again indicating that the mutant and wild-type CP have similar conformations.

Finally, we checked whether the CK2 phosphorylation-deficient mutant was able to form virus particles *in vivo*. For this purpose, *N. benthamiana* leaves were bombarded with the GFP-tagged PVA having three potential CK2 phosphorylation sites in the CP substituted by Ala residues. At 7 days after inoculation, plant tissue surrounding single fluorescent cells was excised carefully and analyzed by immunocapture RT-PCR (Fedorkin et al., 2000) using monoclonal anti-PVA antibodies and virus-specific primers. Consistent with the formation of virus-like particles in bacteria, a single RT-PCR product of the correct size was obtained, indicating that the cells contained viral RNA encapsidated by the mutant CP (see supplemental data online). From this result, we concluded that although the CK2 phosphorylation-negative mutations strongly affect cell-to-cell movement, they do not completely prevent virion formation in single bombarded cells.

Effect of CK2 Phosphorylation on the RNA Binding Activity of PVA CP

Previous results from this laboratory have shown that PVA CP binds single-stranded RNA efficiently but with no sequence specificity (Merits et al., 1998). In a more recent study, we demonstrated that *in vitro* phosphorylation of PVA CP by the plant Ser/Thr protein kinase activity inhibits binding to single-stranded RNA (Ivanov et al., 2001). The results presented above indicate that CK2 is involved in the phosphorylation of PVA CP. Therefore, we next sought to determine whether the affinity of PVA CP for RNA changes upon CK2 phosphorylation. For this purpose, two experimental approaches based on gel retardation (electrophoretic mobility shift assay) and UV cross-linking were used. In the first approach, increasing amounts of PVA CP were incubated in kinase buffer containing unlabeled GTP with or without the addition of the purified rmCK2 α . The kinase reactions were allowed to proceed, and two identical electrophoretic mobility shift assays were performed to compare the binding of nonphosphorylated and phosphorylated PVA CP to the radiolabeled RNA transcript.

The results shown in Figure 9A demonstrate that the mobility transition from free to retarded RNA occurred at a lower concentration of the nonphosphorylated PVA CP (top) compared with that of the protein incubated with rmCK2 α (bottom). These data suggested that the nonphosphorylated PVA CP exhibited higher affinity for RNA than the mixture of CK2-phosphorylated and nonphosphorylated protein. To verify these results, UV cross-linking experiments were performed. First, we incubated unlabeled, nonphosphorylated PVA CP with the ^{32} P-labeled RNA transcript and subjected the mixture to UV irradiation. The unbound RNA was removed by digestion with RNase, and the cross-linked complexes were analyzed by SDS-PAGE and autoradiography. The labeled band with a slower mobility than that of free PVA CP was detected in the position expected for the protein covalently attached to a small number of nucleotides (Figure 9B, lane 3). By contrast, when the ^{32} P-labeled, CK2-phosphorylated PVA CP was

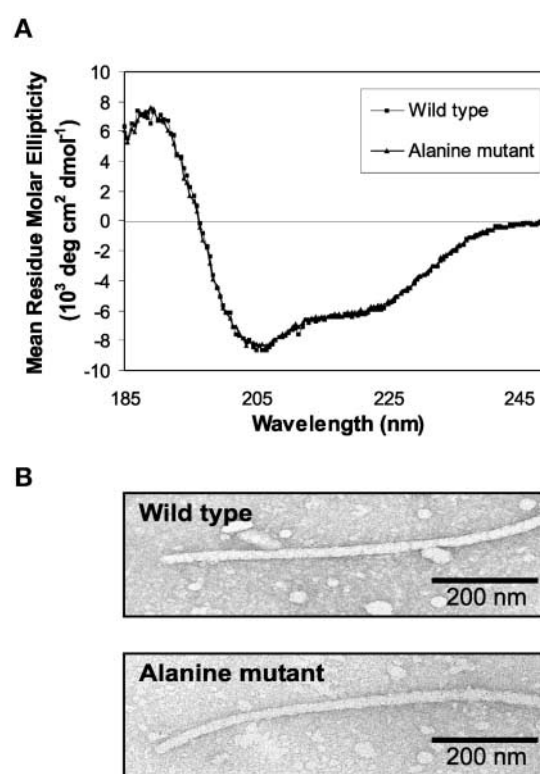


Figure 8. Structural Comparison of Wild-Type PVA CP and Its CK2 Phosphorylation-Deficient Ala Mutant.

(A) CD spectra of wild-type PVA CP and its mutant at 0.1 mg/mL in water. Each CD spectrum represents an average of five scans. Background spectra without protein were subtracted. The spectra were recorded at 20°C.

(B) Electron micrographs of the potyvirus-like particles formed in bacterial cells expressing wild-type PVA CP or its Ala mutant.

incubated with the same unlabeled RNA transcript and assayed by UV cross-linking, only one radioactive band corresponding to the free phosphorylated protein was detected (Figure 9B, cf. lanes 1 and 2). The absence of the labeled band with altered mobility indicated that the phosphorylated protein was not in a close molecular association with RNA; therefore, it could not be cross-linked by UV irradiation. Together, the results of gel retardation and UV cross-linking experiments demonstrated that the phosphorylation of PVA CP by CK2 reduces the affinity of the protein for RNA.

DISCUSSION

Protein kinase CK2 is a highly conserved, ubiquitously expressed, acidophilic Ser/Thr kinase present in all eukaryotic cells examined to date (Allende and Allende, 1995). During the past decade, plant CK2 was shown to be involved in different processes, including cell cycle regulation, transcriptional control, proteasome degradation, RNA translation, nuclear transport, and the regulation of circadian rhythms and the light-signal transduction pathway (Riera et al., 2001). In plants, the enzyme is thought to be a heterogeneous complex formed by different isoforms of catalytic

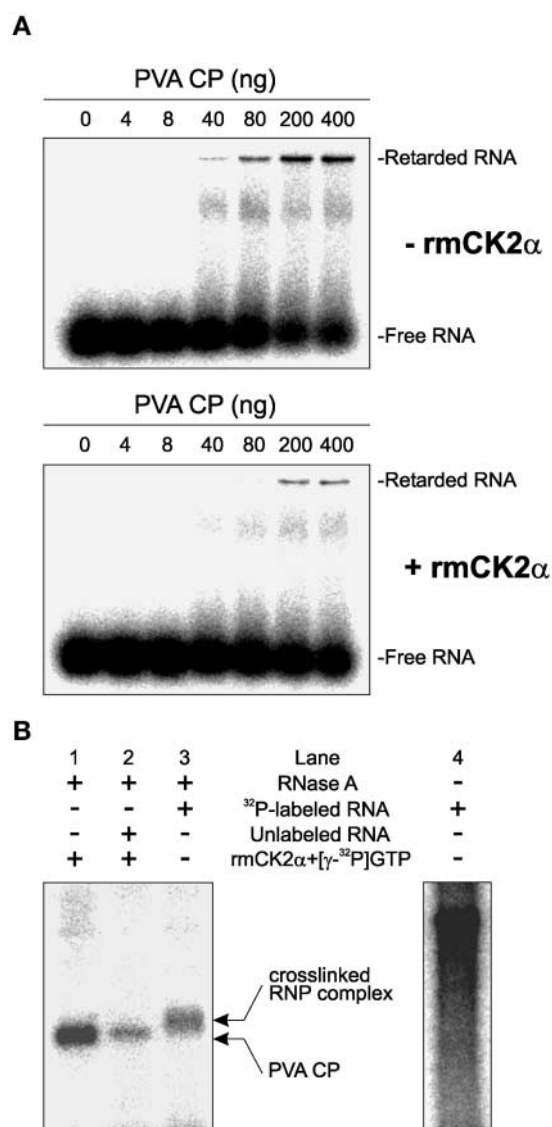


Figure 9. Effect of Phosphorylation by the α -Catalytic Subunit of Recombinant Maize CK2 on the RNA Binding Activity of PVA CP.

(A) Electrophoretic mobility shift assays. Increasing amounts of PVA CP were incubated in kinase buffer with (bottom gel) or without (top gel) the addition of rmCK2 α . The reaction products were incubated with 5 ng of radioactively labeled RNA probe, and protein-RNA complexes were resolved on 1% nondenaturing agarose gels.

(B) Phosphorylated or nonphosphorylated PVA CP was incubated with a synthetic RNA probe and analyzed by UV cross-linking. Before UV irradiation, PVA CP was phosphorylated by rmCK2 α in the presence of γ -³²P-GTP and incubated with unlabeled RNA (lane 2). Alternatively, the same RNA was radioactively labeled and incubated with unlabeled non-phosphorylated PVA CP (lane 3). The resulting protein-RNA complexes, cross-linked by UV irradiation and digested with RNase, were analyzed by SDS-PAGE. Phosphorylated PVA CP was incubated in buffer alone and irradiated with UV light (lane 1). The RNase digestion step was omitted in lane 4. The arrows indicate the positions of the cross-linked protein-nucleotide complex and phosphorylated PVA CP.

(α) and regulatory (β) subunits. The α -subunits are catalytically active by themselves and share >70% sequence identity with CK2 α' -subunits of mammals. By contrast, regulatory β -subunits are catalytically inactive and less conserved between plants and mammals, and their role is to stimulate the catalytic activity of the α -subunit, stabilize the holoenzyme, and regulate its specificity.

The results presented here identify plant CK2 as the kinase responsible for PVA CP phosphorylation. Mutations that affect the CK2 consensus sequence in PVA CP were introduced into the infectious PVA clone and shown to block the cell-to-cell and long-distance movement of the virus. The involvement of acidophilic CK2 in the regulation of the PVA infection cycle was confirmed by the movement-deficient phenotype of the mutant having a substitution of acidic residues for basic Arg residues downstream of the phosphoacceptor site. Furthermore, a spontaneous reversion in the nonphosphorylatable Ala mutant restoring Thr-243 (the second phosphoacceptor site in the triple CK2 consensus sequence) was able to partially rescue virus movement.

A possible mechanism by which CK2 may exert its effect on virus infection could involve the reversible inhibition of PVA CP binding to viral RNA. There are many examples in the literature of phosphorylation by CK2 regulating, either positively or negatively, the protein-nucleic acid interactions (Allende and Allende, 1995; Riera et al., 2001). To understand the mechanism behind such regulation, structural data describing the changes introduced by CK2 phosphorylation into the nucleic acid binding domains of the corresponding proteins are required. Recently, a structural model of PVA CP was proposed on the basis of the experimental data obtained by tritium bombardment combined with theoretical predictions of protein topology (Baratova et al., 2001). According to this model, the CK2 phosphorylation site Thr-242 is located in an interconnecting loop immediately after the β 7 strand (amino acids 235 to 241) inside the structural unit commonly found in RNA binding proteins. Thus, it becomes clear how phosphorylation at Thr-242, which affects the charge and, possibly, the structure of this region, is able to regulate the binding of PVA CP to RNA.

In a previous study, we showed that PVA CP could be phosphorylated in vitro by a plant protein extract only upon particle disassembly (Ivanov et al., 2001). This allowed us to suggest that the CP phosphorylation sites are not exposed on the surface of the virus particle and presumably are located within the core domain involved in the interaction with viral RNA. Here, we show that the CK2 phosphorylation site Thr-242 is located within the C-terminal part of the core domain of PVA CP (Figure 6A). Two viral functions attributed to this domain, virion assembly and cell-to-cell movement, both require CP-RNA interactions; therefore, they can be regulated by CK2 phosphorylation. In agreement with this possibility, the results obtained with GFP-tagged mutant viruses demonstrated that the amino acid substitutions affecting the CK2 consensus sequence in PVA CP produced movement-deficient phenotypes. Interestingly, both hypophosphorylated mutants and mutants mimicking phosphorylation were restricted to single cells, suggesting that a dynamic balance between CP phosphorylation and dephosphorylation is crucial for PVA infectivity.

Our working hypothesis is that phosphorylation of the RNA binding domain of PVA CP by CK2 regulates the amount of viral

template RNA available for replication on the endoplasmic reticulum membranes (Schaad et al., 1997). According to this hypothesis, at early stages of infection, phosphorylation of PVA CP by the endoplasmic reticulum-associated form of CK2 (Faust et al., 2001) prevents premature particle assembly, thereby allowing efficient viral RNA replication to proceed. Alternatively, but not mutually exclusively, phosphorylation of PVA CP may inhibit the formation of nonspecific complexes with cellular RNA when levels of viral RNA are low. Later in the infection cycle, PVA CP binding to viral RNA is restored through dephosphorylation by a yet unidentified protein phosphatase, triggering the activation of the assembly/movement pathway. Importantly, a similar model has been proposed for the regulation of *Rubella virus* encapsidation (Law et al., 2003). Therefore, CP phosphorylation/dephosphorylation may represent a general regulatory mechanism used by both animal and plant viruses.

Recently, the intact virions of another potyvirus, PPV, were shown to interact specifically with anti-phosphoserine and anti-phosphothreonine antibodies (Fernández-Fernández et al., 2002). This finding suggests that in addition to the phosphorylation sites in the core of the CP, which are accessible in the unassembled protein, other phosphorylated residues exist on the virion surface. These particular sites have not been identified in our previous assays with disrupted PVA particles, probably because they already were phosphorylated extensively in vivo. Interestingly, a modification of PPV CP by Ser/Thr-linked *N*-acetylglucosamine also has been reported (Fernández-Fernández et al., 2002). To date, this modification has been found only in those proteins that also are phosphorylated, and there is mounting evidence indicating that the coordinated action of *O*-phosphorylation and *O*-glycosylation plays an important role in controlling fundamental cellular events (Comer and Hart, 2000). Therefore, it is plausible that the interplay between the two post-translational modifications also may regulate the functions of potyviral CPs.

Several lines of evidence indicate that CK2 is not the only enzyme involved in the phosphorylation of PVA CP. These include the incomplete inhibition of PVA CP phosphorylation by heparin in the presence of γ -³²P-ATP in vitro and by DRB in vivo and the presence of several spots on tryptic phosphopeptide maps of the protein phosphorylated by tobacco protein extract. In addition, we have shown that the microbial alkaloid staurosporine, whose potency against CK2 is 1000 times lower than that against other protein kinases, inhibited the phosphorylation of PVA CP in vitro and in vivo (Ivanov et al., 2001). Nevertheless, even a relatively high micromolar concentration of the compound was unable to completely eliminate the phosphorylation of PVA CP. This finding may be explained by a partial inhibition of CK2 while the activity of other protein kinases phosphorylating PVA CP was blocked completely.

Assuming that CP phosphorylation by CK2 is functionally important for the virus, we expect conservation of the CK2 site among potyviral CPs. Indeed, multiple alignment of the amino acid sequences of the CPs of 28 members of the *Potyvirus* genus (Shukla et al., 1994) showed high conservation of the CK2 site. Among the 28 sequences compared, 24 contained Thr or Ser residues within a CK2 consensus motif corresponding to the 242-TTSEED-247 sequence in PVA CP. Another three sequences contained "atypical" CK2 phosphoacceptor sites. In a

recent report, an in vitro DNA transposition-based strategy was used to generate a genome-wide insertion mutant library of PVA (Kekarainen et al., 2002). The virus could not tolerate a five-amino acid insertion at Glu-245, disrupting the CK2 consensus sequence. This finding, together with the results of the current study and data on the conservation of the CK2 motif among potyviral CPs, suggests that the infection cycle of potyviruses requires the recruitment of the cellular protein kinase CK2.

METHODS

Plants and Viruses

PVA strain B11 (Puurand et al., 1994) was propagated in tobacco plants (*Nicotiana tabacum* cv SR1) as described previously (Ivanov et al., 2001). The GFP-tagged cDNA clone of PVA (35S-PVA-GFP^{NIB/CP}) was constructed according to the strategy proposed by Varrelmann and Maiss (2000). Amino acid substitutions that affect the CK2 consensus sequence in PVA CP were introduced into 35S-PVA-GFP^{NIB/CP} using standard molecular biology techniques. The resulting constructs were bombarded into leaf epidermal cells of *Nicotiana benthamiana* using the PDS-1000/He particle delivery system (Bio-Rad Laboratories). GFP expression was visualized using fluorescence microscopy (a Leica MZFLIII microscope [Wetzlar, Germany] equipped with an Olympus DP50 digital camera [Tokyo, Japan]).

Recombinant Protein Expression

All (His)₆/pQE proteins were expressed in *Escherichia coli* strain M15[pREP4] cells and purified using nickel-nitrilotriacetic acid (Ni²⁺-NTA) agarose (Qiagen, Valencia, CA) according to standard protocol. Purification of (His)₆tCK2 α was performed in two steps using chromatography on Ni²⁺-NTA agarose and heparin-Sepharose. Isopropylthio- β -galactoside-induced bacterial cells expressing (His)₆tCK2 α were shaken overnight at 18°C, collected by centrifugation, and resuspended in buffer R1 (100 mM NaCl and 50 mM Tris-HCl, pH 8.0). The suspension was passed three times at ~90 MPa through a prechilled French pressure cell. The lysate was centrifuged at 40,000g for 45 min, and the supernatant fraction was processed by Ni²⁺-NTA agarose chromatography under native conditions. Pooled fractions containing (His)₆tCK2 α were applied onto a heparin-Sepharose column (Amersham Pharmacia Biotech). Proteins were eluted with a linear 0.1 to 1 M NaCl gradient buffered with 50 mM Tris-HCl, pH 8.0. Purified (His)₆tCK2 was stored at +4°C for up to 1 month without detectable loss of activity or protein integrity.

In Vitro Protein Kinase Assays

In vitro phosphorylation assays were performed as described previously (Ivanov et al., 2001) using purified rmCK2 α (70 ng; Calbiochem), partially purified CK2 from tobacco ($\leq$ 50 ng), purified recombinant (His)₆tCK2 α (100 ng), or freshly prepared tobacco protein extract ($\leq$ 200 ng). Reaction mixtures (15 μ L) contained 25 mM Hepes, pH 7.4, 2 mM MnCl₂ or 5 mM MgCl₂, 1.6 μ Ci of γ -³²P-GTP or γ -³³P-ATP (Amersham Pharmacia Biotech), and 0.5 to 1 μ g of purified His-tagged PVA CP, PVA VPg, or TMV MP as a substrate.

In Vivo ³³P-Orthophosphate Labeling and PVA CP Immunoprecipitation

Phospholabeling and immunoprecipitation of PVA CP were performed as described previously (Ivanov et al., 2001). To assess the in vivo effect

of the CK2 inhibitor 5,6-dichloro-1-(β -D-ribofuranosyl)benzimidazole (DRB; Calbiochem) on PVA CP phosphorylation, the compound was added at a final concentration of 500 μ M before vacuum-infiltration of ^{33} P-orthophosphate into the leaf tissue. A stock solution of DRB was prepared in DMSO; therefore, DMSO was present in the phospholabeling solution at a final concentration of 0.5% (v/v). PVA CP was precipitated with a sheep anti-PVA polyclonal antibody (Scil Diagnostics, Martinsried, Germany). NET buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 50 mM NaF, 5 mM EDTA, pH 8.0, 0.5% [v/v] Nonidet P-40, and 0.02% sodium azide) was supplemented with 2% BSA to reduce nonspecific antibody binding. Protein G-Sepharose (Amersham Pharmacia Biotech) was used as an immunoadsorbent. Antibodies were first prebound to protein G-Sepharose and then incubated with the cell lysate containing PVA CP. This modification allowed the removal of unbound antibodies from the incubation mixture before the addition of the antigen.

Immunofluorescence Staining

Tobacco protoplasts were harvested at 9 days after inoculation and processed for indirect immunofluorescence. The protoplasts were fixed for 80 min in 4% paraformaldehyde, 0.2% glutaraldehyde (electron microscopy grade; Sigma), and 500 mM mannitol in 0.5% Mes, pH 5.7. Fixed cells were washed three times with Dulbecco's medium containing 0.2% BSA, incubated for 20 min in 0.1% NaBH₄ in PBS, and permeabilized for 20 min with 0.1% Triton X-100 in PBS. To inhibit nonspecific antibody binding, cells were incubated for 90 min in 5% BSA and 0.1% bovine gelatin in Dulbecco's medium. Protoplasts were incubated overnight with primary antibodies (rabbit polyclonal anti-tCK2 α and mouse monoclonal anti-PVA CP) in Dulbecco's medium at +4°C. After three washes, cells were incubated for another 5 h with goat anti-rabbit or anti-mouse secondary antibodies conjugated with Alexa Fluor 594 or Alexa Fluor 488 (Molecular Probes, Eugene, OR). After six washes, the protoplasts were analyzed using a Bio-Rad MRC-1024 confocal microscope.

Tryptic Phosphopeptide Mapping

After 12% SDS-PAGE, radiolabeled proteins were transferred electrophoretically to a polyvinylidene difluoride membrane (Immobilon-P; Millipore, Bedford, MA). The protein bands were visualized by Ponceau S staining and autoradiography, excised from the membranes, cut into small pieces, and soaked for 30 min at 37°C in 0.5% polyvinylpyrrolidone-360 (Sigma) in 100 mM acetic acid. Membrane pieces were washed with water and immersed completely in 50 μ L of digestion buffer (50 mM NH₄HCO₃ in 10% [v/v] acetonitrile) containing 0.05 μ g/ μ L trypsin (Sequencing-Grade Modified Trypsin; Promega). The samples were digested first for 4 h at 37°C and then overnight at the same temperature after a second addition of trypsin. The digests containing tryptic peptides released from the membranes were diluted with deionized water, concentrated in a centrifugal vacuum concentrator, cleaned by repeated lyophilization, resuspended in 5 μ L of pH-1.9 buffer (0.58 M formic acid and 1.36 M glacial acetic acid), and spotted onto 20- $\times$ 20-cm cellulose thin layer chromatography (TLC) plates (VWR International, West Chester, PA).

Peptides were separated in the first dimension using the Hunter thin layer electrophoresis system (HTLE-7000; C.B.S. Scientific, Del Mar, CA). Electrophoresis at 2000 V for 40 min in pH-1.9 buffer in the first dimension was followed by ascending chromatography in 39% *n*-butanol, 30% pyridine, and 6% glacial acetic acid (v/v) in the second dimension. The plates were air-dried, and the positions of phosphopeptides were detected using a phosphor imager (Fuji Photo Film, Tokyo, Japan). Individual phosphopeptides were eluted from the plates in pH-1.9 buffer, lyophilized, and subjected to two-dimensional phosphoamino acid analysis or automated Edman degradation as described previously (Blume-Jensen et al., 1995). Briefly, Edman degradation was performed using an

Applied Biosystems Gas Phase Sequencer model 477A after coupling of phosphopeptides to Sequelon-AA membranes (Millipore). Released phenylthiohydantoin derivatives from each cycle were spotted onto TLC plates, and radioactivity was quantified using a phosphor imager.

Phosphoamino Acid Analysis

Phosphoamino acid analysis was performed essentially as described by Boyle et al. (1991). After the efficiency of phosphopeptide elution from cellulose was checked by liquid scintillation counting, the eluted material was lyophilized twice and subjected to hydrolysis in 6 N HCl for 30 min at 110°C. Samples were lyophilized again, resuspended in 5 μ L of pH-1.9 buffer containing 0.6 μ g/ μ L each of nonlabeled phosphoamino acid standards (Sigma), and spotted onto a 20- $\times$ 20-cm TLC cellulose plate. Thin layer electrophoresis was performed at 1600 V for 40 min in pH-1.9 buffer in the first dimension and at 1400 V for 20 min in pH-3.5 buffer (0.5% [v/v] pyridine and 0.87 M glacial acetic acid) in the second dimension. Phosphoamino acid standards were visualized by spraying with 0.25% (w/v) ninhydrin solution in acetone and developed at 80°C. The positions of radioactive phosphoamino acids were detected using a phosphor imager.

Reverse-Phase Chromatography and Mass Spectrometry

PVA CP was phosphorylated using tobacco protein extract in the presence of γ - ^{33} P-ATP as described above. For in-liquid digestion, the protein first was desalted by reverse-phase chromatography on a 0.21- $\times$ 10-cm C1 column (TSK-TMS250; TosohHaas, Tokyo, Japan). The column was developed using a linear gradient of 3 to 100% (v/v) acetonitrile in 0.1% (v/v) trifluoroacetic acid over 60 min at a flow rate of 200 μ L/min. Fractions containing radioactivity were pooled, lyophilized, and dissolved in digestion buffer (100 mM NH₄HCO₃ in 10% [v/v] acetonitrile). Trypsin (Sequencing-Grade Modified Trypsin; Promega) was added at a final concentration of 0.05 μ g/ μ L, and digestion was performed at 37°C overnight. The digested peptides were separated by micro-reverse-phase chromatography on a 0.3- $\times$ 150-mm PepMap C18 column (LC Packings, Amsterdam, The Netherlands) at a flow rate of 2 μ L/min. Elution was performed using a linear gradient (5 to 50% [v/v] in 160 min) of acetonitrile in 0.1% (v/v) formic acid, fractions were collected, and the radioactive material was located by liquid scintillation counting. The fraction with the highest label content was analyzed on a Biflex matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometer (Bruker-Daltonics, Billerica, MA) in a positive ion reflector mode using α -cyano-4-hydroxycinnamic acid as a matrix. MALDI spectra were calibrated with angiotensin II and adrenocorticotropin 18-39.

Liquid Chromatography-Tandem Mass Spectrometry Analysis

A Coomassie Brilliant Blue R 250-stained protein band was cut out of a 12% SDS-polyacrylamide gel and destained. Proteins were reduced with DTT and alkylated with iodoacetamide before overnight digestion with trypsin (Sequencing-Grade Modified Trypsin; Promega). The peptides were extracted once with 25 mM NH₄HCO₃ and twice with 5% formic acid, and the extracts were pooled. The peptides were separated by microbore reverse-phase HPLC on a 0.075- $\times$ 150-mm PepMap column (LC Packings) at a flow rate of 0.25 μ L/min. Chromatography was performed with a linear gradient of acetonitrile in 0.1% formic acid, and the eluate was injected directly into a Quadrupole-TOF mass spectrometer (Micromass, Manchester, UK) equipped with an electrospray ionization source. Tandem mass spectrometry (MS/MS) spectra were acquired by colliding the doubly charged precursor ions with argon collision gas with accelerating voltages of 30 to 45 V. Database searches were performed using the Mascot MS/MS ion search (<http://www.matrixscience.com/>).

Purification of the PVA CP Kinase from Tobacco

All procedures were performed at 4°C. The kinase was isolated by a modification of a previously published procedure (Klimczak et al., 1992). Tobacco leaves (100 g) were homogenized in 120 mL of buffer BM (50 mM Tris-HCl, pH 7.5, 5 mM NaF, 5 mM EDTA, 0.3 mM phenylmethylsulfonyl fluoride, and 20% [v/v] glycerol) and centrifuged at 10,000g for 30 min. The 10,000g supernatant was centrifuged once again at 100,000g for 12 h, and the soluble material was purified by batch adsorption on 10 mL of heparin-Sepharose 6 Fast Flow (Amersham Pharmacia Biotech). The batch was washed six times with 45 mL of buffer CM (50 mM Tris-HCl, pH 7.5, 5 mM 2-mercaptoethanol, and 5% [v/v] glycerol) to remove unbound proteins and poured into a column. The column was washed with buffer CM, and the absorbance of the eluate was monitored until the A_{280} had returned to baseline. The column was eluted with 100 mL of a linear gradient of 0 to 1.5 M KCl in buffer CM. Fractions of 4 mL were collected and dialyzed overnight against a 50-fold excess of buffer CM, and 5- μ L aliquots were assayed for the phosphorylation of PVA CP. Fractions containing the highest PVA CP kinase activity were examined by in-gel kinase assays, which were performed as described previously (Zhang et al., 1998) except that the reaction buffer (20 mM Hepes, pH 7.4, and 2 mM $MnCl_2$) contained 50 μ Ci of γ - ^{32}P -GTP.

Gel Retardation Assay (Electrophoretic Mobility Shift Assay)

Gel retardation assays were performed with radiolabeled synthetic RNA probe and unlabeled protein (Li and Palukaitis, 1996). Phosphorylation reactions were performed in a final volume of 5 μ L containing 25 mM Hepes, pH 7.4, 1 mM $MnCl_2$, 400 μ M GTP, increasing amounts of recombinant PVA CP, and rmCK2 α (70 ng). After incubation, the reaction products were transferred to fresh tubes containing 5 μ L of RNA binding buffer (10 mM Hepes, pH 7.4, 1 mM EDTA, 50 mM NaCl, 1 mM DTT, 1 mg/mL BSA, and 10% [v/v] glycerol) plus 5 ng of the radioactively labeled RNA transcript corresponding to the 5' untranslated region of PVA RNA synthesized as described by Merits et al. (1998). The mixtures were incubated on ice for 30 min and subjected to electrophoresis on 1% (w/v) nondenaturing agarose gels in TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0).

UV Cross-Linking/Label-Transfer Assay

In vitro phosphorylation of PVA CP was performed using rmCK2 α , and the following incubation mixtures were prepared for UV cross-linking. One mixture contained radiolabeled phosphorylated PVA CP and 500 ng of unlabeled RNA transcript synthesized as described previously (Merits et al., 1998). Alternatively, 5 ng of the same ^{32}P -labeled RNA transcript was incubated with unlabeled nonphosphorylated PVA CP. In a control experiment, radiolabeled phosphorylated PVA CP was incubated without the addition of RNA. UV cross-linking was performed as described previously (Daròs and Carrington, 1997).

Electron Microscopy

Bacterial cells from 1.5 mL of induced culture expressing wild-type PVA CP or its Ala mutant were resuspended in 0.5 mL of buffer L (20 mM Hepes, pH 7.6, 6 mM $MgCl_2$, 100 mM NaCl, and 16% sucrose [w/v]). Ten microliters of 50 mg/mL lysozyme was added, and the mixture was incubated for 5 min at room temperature followed by 15 min on ice. Cells were stored at -80°C overnight, thawed in an ice-water bath, and centrifuged (16,000g for 20 min at 4°C). The supernatants were applied to Formvar carbon-coated grids and negatively stained with 2% (w/v) neutral uranyl acetate. Images were taken on a JEOL 1200 EX II electron microscope at 80 kV.

Upon request, materials integral to the findings presented in this publication will be made available in a timely manner to all investigators on similar terms for noncommercial research purposes. To obtain materials, please contact K. Mäkinen, kristiina.makinen@helsinki.fi.

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