



## Phosphorylation of the TGBp1 movement protein of *Potato virus X* by a *Nicotiana tabacum* CK2-like activity

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### ABSTRACT

The movement protein (MP) TGBp1 of the potexvirus *Potato virus X* (PVX) is a multifunctional protein required for cell-to-cell movement within the host plant. Recent work on other plant viruses has indicated that MP phosphorylation by host kinases can regulate MP function. In this study, we demonstrate that recombinant and native TGBp1 are phosphorylated by *Nicotiana tabacum* extracts from both PVX-infected and non-infected leaves. The phosphorylation activity present in plant extracts has distinctive characteristics of casein kinase 2 (CK2): it is inhibited by heparin, stimulated by polylysine, and uses either ATP or GTP as phosphoryl donors. We also demonstrate that TGBp1 is efficiently phosphorylated by recombinant tobacco CK2  $\alpha$  subunit and by partially purified tobacco CK2. Phosphopeptide mass mapping reveals that TGBp1 is phosphorylated in Ser-165, which is localized within a CK2 consensus sequence. Our results strongly suggest that a *N. tabacum* kinase of the CK2 family is involved in TGBp1 phosphorylation during the course of viral infection.

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### 1. Introduction

*Potato virus X* (PVX) is the type member of the *Potexvirus* genus. Its genome encodes the viral replicase, three movement proteins (TGBp1, TGBp2 and TGBp3, collectively referred as the “triple gene block”) and the viral coat protein (CP) (Huisman et al., 1988; Orman et al., 1990). PVX has been extensively used as a model system for studies on virus replication, cell-to-cell movement, and virus-induced gene silencing (Batten et al., 2003; Verchot-Lubicz, 2005). The main role of movement proteins (MPs) resides in assisting the viral progeny in cell-to-cell and in long-distance spreading through plant tissues. Cell-to-cell movement involves formation of an MP-viral RNA complex which interacts with host proteins to increase the plasmodesmal size exclusion limit and to potentiate its own transport into neighboring cells (Lucas, 2006; Verchot-Lubicz et al., 2007).

In PVX, the three MPs and the CP are essential for viral mobilization (Chapman et al., 1992; Morozov et al., 1997). PVX TGBp1 is a multifunctional protein with RNA helicase (Kalinina et al., 2002) and suppressor of post-transcriptional gene silencing activities (Voinnet et al., 2000), being involved in cell-to-cell movement and plasmodesmal gating (Yang et al., 2000; Howard et al., 2004).

In recent years, it is becoming evident that phosphorylation by host kinases may regulate the function of plant viral proteins. In the case of PVX, it has been shown that the CP can be phosphorylated by protein kinase C (PKC), a mixture of casein kinases 1 and 2 (CK1 and CK2) and a soluble kinase from *Nicotiana glutinosa* (Atabekov et al., 2001). The phosphorylation of PVX CP leads to the translation of the encapsidated viral RNA *in vitro*, suggesting that CP phosphorylation regulates the translational activity of PVX in primary inoculated cells (Atabekov et al., 2001). In contrast, phosphorylation of the PVX MP has not yet been demonstrated.

In this work, we present the first report on potexviral MP phosphorylation by showing that recombinant and native PVX TGBp1 is phosphorylated by a host kinase with CK2 characteristics. We also show that recombinant CK2  $\alpha$ -subunit phosphorylates TGBp1 in Ser-165, located in a CK2 consensus phosphorylation site. These results represent a first step to establish the potential role of TGBp1 phosphorylation in the mobilization and replication of PVX.

### 2. Materials and methods

#### 2.1. Plant infection and preparation of plant protein extracts

*Nicotiana tabacum* cv. Xhanti plants were grown in chambers under long day conditions (16 h light/8 h darkness) at 26 °C. To establish systemic infections, plants were mechanically inoculated with PVX (strain CP) using carborundum as an abrasive. PVX-infected tissue (0.5 mg/ml) was ground in 20 mM phosphate

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sodium buffer, pH 7.4, and the extract was used to inoculate intermediate leaves. Fully expanded leaves (approximately 9 cm) of infected (10 days post-infection) and non-infected plants were used to prepare protein extracts. Fresh leaf tissue (500 mg) was homogenized in 1 ml of pre-chilled buffer H (10 mM buffer phosphate, pH 7, 100 mM NaCl, 2 mM 2-mercaptoethanol and 1 mM PMSF). A protease inhibitor cocktail was added at the concentrations recommended by the manufacturer (Sigma–Aldrich). Homogenates were centrifuged at  $1000 \times g$  for 5 min at 4 °C to obtain a crude cell wall pellet and a supernatant. Supernatants were centrifuged at  $15,000 \times g$  for 30 min at 4 °C and stored at –70 °C until use. Cell walls were further purified by homogenization at 4 °C in 10 volumes of buffer H with 2% Triton X-100 and centrifugation at  $1000 \times g$  for 5 min at 4 °C. This procedure was repeated twice, followed by four washes in buffer H without detergent. The resulting insoluble material was resuspended in 0.5 volume of buffer H, and stored at –70 °C until use. Protein concentration was determined according to Bradford (1976) using bovine serum albumin as standard.

## 2.2. Cloning of TGBp1 in bacterial expression vector

The TGBp1 coding sequence was amplified by PCR using as a template a full-length copy of the PVX genome (Orman et al., 1990) and primers GGATCCATATGGATATTCTCATCATTAGTTTG (NdeI site underlined) and GTCGACCTATGCCCCCTGCGCGGACATAAGT (Sall site underlined), complementary to amino acids 1–8 and 220–226, respectively. A 681 bp fragment was cloned into the pZERO-2 vector (Invitrogen) and then subcloned between the NdeI and Sall sites of the bacterial expression vector pET-28a (+) (Novagen) to obtain plasmid pET24. Cloned TGBp1 sequence was confirmed by single-stranded dideoxy DNA automated sequencing.

## 2.3. Cloning of tobacco CK2 $\alpha$ subunit (NtCK2)

The NtCK2 coding sequence was amplified by RT-PCR using as template total RNA extracted from *N. tabacum* leaves. cDNA synthesis was performed using MMLV reverse transcriptase and oligo-dT primer according to manufacturer's recommendations (Invitrogen). About 10 ng of cDNA in 10 mM Tris–HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, 100  $\mu$ M dNTP, 5 U Taq polymerase, and 2  $\mu$ M of each primer were used for PCR amplification. The CK2 sequence was amplified by PCR and primers AAACATATGTCGAAAGCTCGTGTTCACACC (NdeI site underlined) and AAACCCGGGCTACTGCTCCGCATCCTGCTATTC (SmaI site underlined) designed on the basis of published tobacco sequences (Genbank AJ438263 and AJ438264). The 1002 bp-PCR fragment was cloned into pGEM-T Easy vector (Promega) and its sequence was confirmed by single-stranded dideoxy DNA automated sequencing and deposited in Genbank under accession number EU555192. The NtCK2 cDNA was subcloned into of the bacterial expression vector pET-28a (+) (Novagen) to obtain plasmid pETCK2.

## 2.4. Expression of recombinant TGBp1 and CK2 $\alpha$ subunit

Plasmids pETp24 and pETCK2, allowing expression of TGBp1 and CK2  $\alpha$  subunit as N-terminal histidine-tagged proteins, respectively, were transformed into *Escherichia coli* strain BL21 (DE3) following standard procedures. Transformants were individually grown in 3 ml of LB medium containing 50  $\mu$ g/ml kanamycin at 37 °C overnight. Pre-cultures were then inoculated into 500 ml of fresh LB-kanamycin medium and further grown until OD<sub>600</sub> = 0.6. At this point, recombinant protein expression was induced by addition of 1 mM IPTG and the incubation was continued for 3 additional hours. After harvesting of bacteria, purification of the recombinant protein was performed in denaturing conditions for TGBp1 and

non-denaturing conditions for CK2 according to the manufacturer's instructions (Novagen).

## 2.5. CK2 purification by heparin–sepharose

Purification was carried out by immobilized metal affinity chromatography according to the protocol described by Klimczak et al. (1995). Tobacco leaves (30 g) were homogenized in a mortar with BM buffer (50 mM Tris–HCl, pH 7.4, 5 mM NaF, 5 mM EDTA, 0.3 mM PMSF and 20% (v/v) glycerol) and the extract was centrifuged for 30 min at  $10,000 \times g$ . The complete procedure was performed at 4 °C. The supernatant was applied in batch adsorption onto 10 ml of heparin–sepharose for 1 h and washed five times with 40 ml of CM buffer (50 mM Tris–HCl, 5 mM 2-mercaptoethanol, and 5% (v/v) glycerol, pH 7.4). The batch was poured into a column, washed once again with buffer CM, and eluted with 80 ml of a linear gradient of 0–1.5 M NaCl in the same buffer. Fractions of 2.5 ml were collected and 10  $\mu$ l were assayed for the phosphorylation of recombinant TGBp1.

## 2.6. Recombinant TGBp1 phosphorylation assays

Phosphorylation assays were performed using purified recombinant NtCK2 (200 ng per reaction), partially purified *N. tabacum* CK2 (300 ng per reaction), or freshly prepared *N. tabacum* protein extracts (1  $\mu$ g per reaction). Reaction mixtures (30  $\mu$ l) contained 20 mM Tris–HCl, pH 8.2, 5 mM MnCl<sub>2</sub> or 5 mM MgCl<sub>2</sub> and 10  $\mu$ Ci of  $\gamma$ -<sup>32</sup>P-ATP (6,000 Ci/mmol; New England Nuclear) or  $\gamma$ -<sup>32</sup>P-GTP (5,000 Ci/mmol; Amersham Biosciences). Reactions were carried out in the presence or absence of heparin, polylysine, unlabelled ATP or GTP at the concentrations indicated in the figures, and 0.5–1  $\mu$ g of purified his-tagged TGBp1 as substrate. A phosphatase inhibitor mixture was used at the concentrations recommended by the manufacturer (Sigma–Aldrich). Reactions were stopped by adding 5  $\mu$ l of 250 mM EDTA and 10  $\mu$ l of SDS-sample buffer, followed by boiling for 3 min, and analyzed by SDS-PAGE. Localization of radiolabelled proteins was accomplished by autoradiography.

## 2.7. Native TGBp1 phosphorylation assays

Protein extracts from PVX-infected and non-infected plant tissues were prepared as described above. Soluble extracts (approximately 400  $\mu$ g of total protein) were incubated during 10 min at 28 °C in the presence of 5 mM MgCl<sub>2</sub> and 100  $\mu$ Ci of  $\gamma$ -<sup>32</sup>P-ATP (6,000 Ci/mmol; New England Nuclear) alone or in the presence of 100  $\mu$ M cold ATP, 100  $\mu$ M cold GTP or 10  $\mu$ M polylysine. Reactions were stopped with 10 mM EGTA and subsequently immunoprecipitated with polyclonal TGBp1-specific antibodies or pre-immune serum as a control.

Immunoprecipitated proteins were resolved in 15% SDS-PAGE and electroblotted onto nitrocellulose membranes. Membranes were probed with TGBp1-specific antiserum or a commercial PVX capsid-specific antiserum (Agdia) as a control and subjected to autoradiography.

## 2.8. Liquid chromatography–tandem mass spectrometry analysis

PVX MP was phosphorylated using recombinant NtCK2  $\alpha$  subunit in a reaction mixture containing 20 mM Tris–HCl, pH 8.2, 5 mM MgCl<sub>2</sub> and 50  $\mu$ M ATP. The reaction mixture was separated in a 15% SDS-PAGE and the specific Coomassie Brilliant Blue R 250-stained protein band was cut out of the gel and destained. Mass Spectrometry analysis was made by the Scripps Center for Mass Spectrometry (La Jolla, CA) obtaining a sequence coverage of 72%.

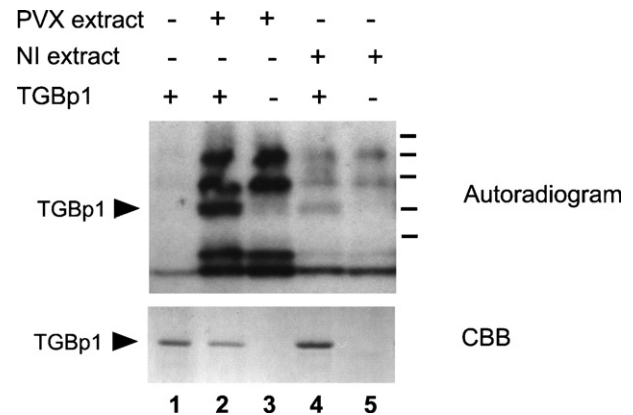
### 2.9. RNA extraction and Northern blot assay

Total RNA was extracted from PVX-infected and non-infected leaves using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. RNA was denatured with formaldehyde and samples (10  $\mu$ g) were resolved in agarose gel electrophoresis, transferred to a nylon membrane, dried at 80 °C and UV-crosslinked. Membranes were hybridized with probes for NtCK2 cDNA or 17s ribosomal RNA labeled with  $\alpha$ - $^{32}$ P-CTP (Prime-a-gene Labeling System, Promega) according to the manufacturer's instructions. Prehybridization, hybridization and washing were performed with 0.1 $\times$  SSC solution, containing 0.2% SDS. Radioactive bands were visualized with a phosphoimager scanner (Storm 820 Molecular Dynamics, Amersham Biosciences) and quantified with ImageQuant Software (Molecular Dynamics, Amersham Biosciences).

## 3. Results

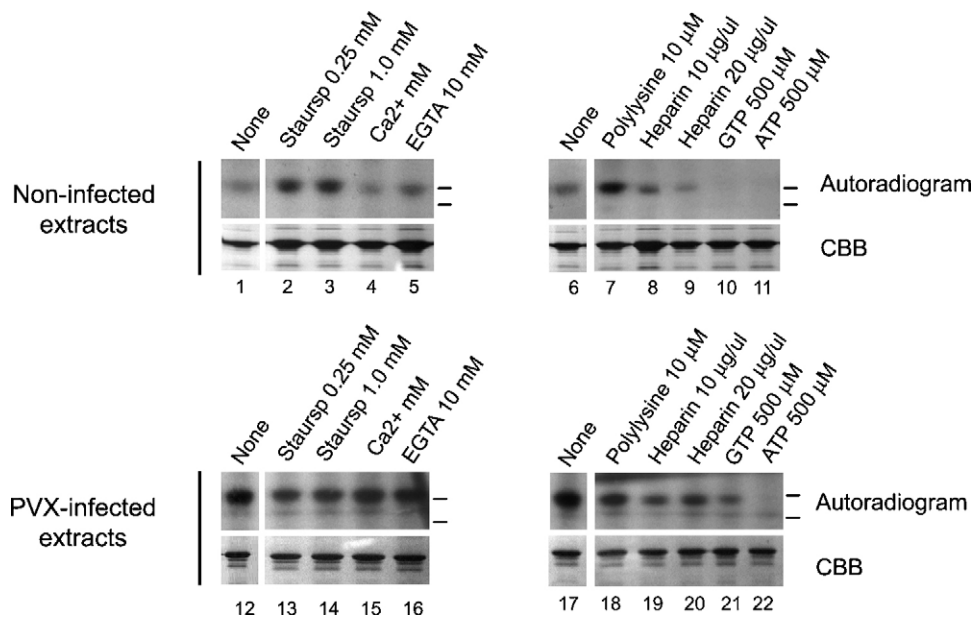
### 3.1. In vitro phosphorylation of PVX TGBp1 by cellular kinases present in PVX-infected and non-infected *N. tabacum* plants

PVX TGBp1 was expressed in bacteria as a fusion protein with an N-terminal histidine tag and was purified to homogeneity. Plant extracts were fractionated to assay the kinase activity of soluble and cell wall-enriched fractions. Phosphorylation reactions were performed in the presence of [ $\gamma$ - $^{32}$ P]-ATP using purified recombinant TGBp1 and protein extracts from either PVX-infected or non-infected *N. tabacum* plants. Phosphorylation products were separated by SDS-PAGE, transferred to nitrocellulose membranes and autoradiographed. As shown in Fig. 1 (lanes 2 and 4), recombinant TGBp1 was specifically phosphorylated by a kinase activity present in soluble extracts of both virus-infected and non-infected plants. Phosphorylation of TGBp1 was 3 to 6 times higher when using extracts from PVX-infected plants. This result cannot be attributed to extra TGBp1 produced by viral infection, since control phosphorylation assays performed with viral-infected extract



**Fig. 1.** PVX TGBp1 phosphorylation by cellular kinases present in non-infected and PVX-infected tobacco plant extracts. Phosphorylations were carried out with [ $\gamma$ - $^{32}$ P]-ATP and 5 mM MgCl<sub>2</sub> and the products of reaction mixes were resolved in 15% SDS-PAGE, stained with Coomassie Brilliant Blue (CBB; lower panel), and autoradiographed (upper panel). Lane 1: recombinant TGBp1 in the absence of plant extracts. Lanes 2 and 4: recombinant TGBp1 incubated with non-infected (NI) or PVX-infected *N. tabacum* plant extracts. Lanes 3 and 5: incubations without recombinant TGBp1 of non-infected and PVX-infected plant extracts, respectively. Molecular mass markers (61, 47.8, 37, 24.5 and 19.2 kDa) are indicated on the right side of the autoradiograph.

and without recombinant TGBp1 do not show a phosphorylation band of the right molecular weight (Fig. 1, lane 3), indicating that the amount of native TGBp1 is too low to be detected (see below). In contrast, other radioactive bands corresponding to endogenous kinase substrates were detected in the absence of TGBp1 (Fig. 1, lanes 3 and 5). No phosphorylation was detected when recombinant TGBp1 was incubated alone with the radiolabeled donor (Fig. 1, lane 1), confirming that recombinant TGBp1 cannot bind [ $\gamma$ - $^{32}$ P]-ATP by itself. In addition, no specific phosphorylation could be detected when cell wall-enriched extracts were used as enzymatic source (data not shown).



**Fig. 2.** In vitro phosphorylation of PVX TGBp1 by a tobacco CK2-like kinase. Purified recombinant PVX TGBp1 was incubated with non-infected (upper panels) or PVX-infected (lower panels) *N. tabacum* plant extracts and [ $\gamma$ - $^{32}$ P]-ATP in the presence of 0.25 mM (lanes 2 and 13) and 1 mM (lanes 3 and 14) staurosporin, 2 mM Ca<sup>2+</sup> (lanes 4 and 15), 10 mM EGTA (lanes 5 and 16), 10  $\mu$ M polylysine (lanes 7 and 18), 10  $\mu$ g/ml (lanes 8 and 19) and 20  $\mu$ g/ml heparin (lanes 9 and 20), 500  $\mu$ M GTP (lanes 10 and 21), 500  $\mu$ M ATP (lanes 11 and 22) or without additions (lanes 1 and 12). Products of reactions mixes were resolved in 15% SDS-PAGE, stained with Coomassie Brilliant Blue (CBB; lower lanes), and autoradiographed (upper lanes). Molecular mass markers (24.5 and 19.2 kDa) are indicated on the right side of the autoradiographs.

Thus, we conclude that recombinant TGBp1 is phosphorylated by a soluble kinase activity present in tobacco cells.

### 3.2. *In vitro* phosphorylation of recombinant TGBp1 by a CK2-like activity

To characterize the properties of the kinase(s) responsible for TGBp1 phosphorylation, further assays were performed with the addition of different stimulatory and inhibitory factors. To determine whether a  $\text{Ca}^{2+}$ -dependent protein kinase was involved, phosphorylation assays were conducted in the presence of either EGTA or  $\text{Ca}^{2+}$ . No stimulation was observed in the presence of 2 mM  $\text{Ca}^{2+}$  when non-infected or PVX-infected extracts were used (Fig. 2, lanes 4 and 15, respectively). Similarly, EGTA was unable to inhibit TGBp1 phosphorylation irrespectively of which plant extract was used in the reaction mix (Fig. 2, lanes 5 and 16). These results ruled out the involvement of  $\text{Ca}^{2+}$ -dependent protein kinases, like PKC.

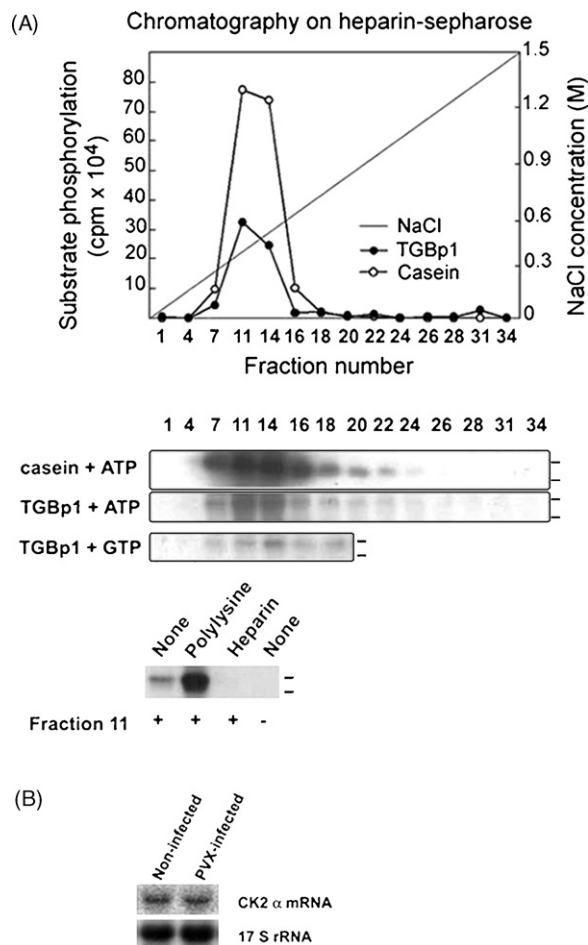
TGBp1 phosphorylation was also conducted in the presence of staurosporine, a powerful protein kinase inhibitor of broad specificity (Ruegg and Burgess, 1989). Even though this inhibitor is usually effective in the nanomolar range, TGBp1 phosphorylation by non-infected or PVX-infected extracts was not inhibited by the presence of 0.25 and 1 mM staurosporine (Fig. 2, lanes 2 and 3, and 13 and 14, respectively). This indicates that TGBp1 phosphorylation might be mediated by a protein kinase(s) partially resistant to staurosporine, such as CK1 or CK2 (Meggio et al., 1995). Surprisingly, in non-infected extracts a stimulation of TGBp1 phosphorylation can be observed (Fig. 2, compare lanes 2 and 3 to lane 1). These results may indicate that the phosphorylation of TGBp1 is indirectly regulated by a staurosporine-sensitive kinase.

To assess the possible involvement of CK1 and CK2 kinases, phosphorylation assays were supplemented with heparin, a specific inhibitor of both kinase families (Vaglio et al., 1996; Guerra et al., 1999). As shown in Fig. 2, TGBp1 phosphorylation by non-infected (lanes 8 and 9) and PVX-infected (lanes 19 and 20) plant extracts was partially inhibited by heparin. To distinguish between CK1 and CK2, we tested the unique ability of CK2 to use both ATP and GTP as phosphoryl donors (Wang et al., 1992; Allende and Allende, 1995; Antonelli et al., 1996; Niefind et al., 1999). *In vitro* phosphorylation of TGBp1 with non-infected and PVX-infected plant extracts was carried out in the presence of [ $\gamma$ - $^{32}\text{P}$ ]-ATP and competed by 500  $\mu\text{M}$  of either GTP or ATP. As shown in Fig. 2, addition of cold GTP completely competed out recombinant TGBp1 phosphorylation by non-infected extracts (Fig. 2, lane 10) and partially inhibited phosphorylation by PVX-infected extracts (Fig. 2, lane 21). On the other hand, TGBp1 phosphorylation by both plant extracts was efficiently competed out when cold ATP was added (Fig. 2A, lanes 11 and 22).

Another characteristic property of CK2 is its stimulation by polylysine (Leroy et al., 1995; Meggio and Pinna, 2003). As shown in Fig. 2, treatment with polylysine increased the relative phosphorylation of recombinant TGBp1 by non-infected extracts by threefold to fourfold (Fig. 2, lane 7). In contrast, no increase was observed when using PVX-infected extracts (Fig. 2, lane 18). This may be due to the fact that, under these experimental conditions, basal levels of TGBp1 phosphorylation are already very high, masking an increase in the intensity of the band.

Taken together, heparin inhibition, polylysine stimulation and, especially, GTP competition suggest that TGBp1 is phosphorylated by a CK2-like kinase present in tobacco extracts.

To obtain more information on the kinase involved in TGBp1 phosphorylation, we partially purified the TGBp1-kinase activity from non-infected plants. Heparin-sepharose was chosen to this aim, since it is well known that it strongly binds CK2 (Klimczak et

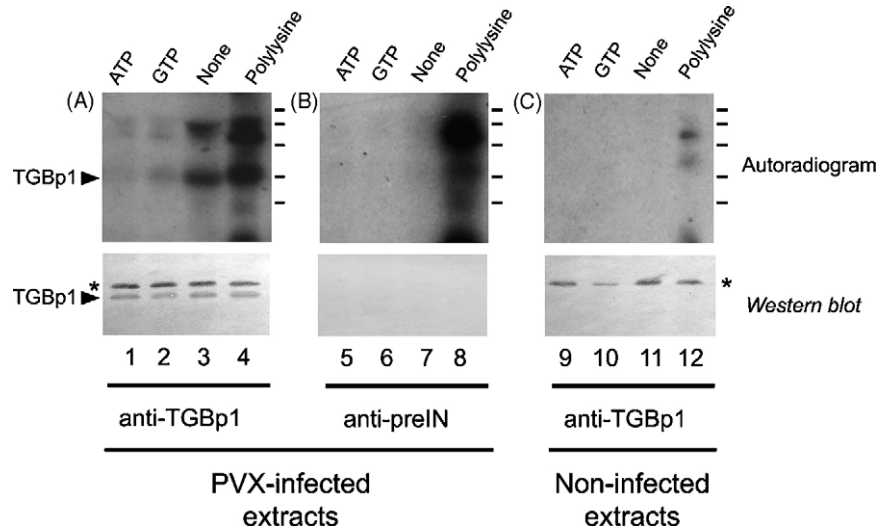


**Fig. 3.** (A) *In vitro* TGBp1 phosphorylation by a partially purified *N. tabacum* CK2 activity. Upper panel: elution profile of kinase activity from a heparin-sepharose column. Kinase activity was determined using casein (○) or TGBp1 (●) as substrates. Middle panel: phosphorylation assays of casein and TGBp1 in the presence of [ $\gamma$ - $^{32}\text{P}$ ]-ATP or [ $\gamma$ - $^{32}\text{P}$ ]-GTP in the fractions collected from the heparin-sepharose column. Fractions 11–14 correspond to eluates at 400–500 mM NaCl. Lower panel: further characterization of fraction 11 kinase activity by recombinant TGBp1 phosphorylation in the presence of heparin or polylysine. Molecular mass markers (24.5 and 19.2 kDa) are indicated on the right side of the autoradiographs. (B) Northern blot analysis of total RNA extracted from non-infected and PVX-infected plants. Membranes were blotted with [ $^{32}\text{P}$ ]-labelled probes specifically recognizing NtCK2A transcripts (upper lanes) and 17 S ribosomal RNA (lower lanes).

al., 1995). The TGBp1-kinase activity eluted from the column as a major peak between 400 and 500 mM NaCl (Fig. 3A, upper panel) and co-eluted with a kinase activity that phosphorylates casein, a well-known CK2 substrate (Fig. 3A, upper and middle panel). The purified activity was able to phosphorylate recombinant TGBp1 in the presence of [ $\gamma$ - $^{32}\text{P}$ ]-ATP and also [ $\gamma$ - $^{32}\text{P}$ ]-GTP (Fig. 3A, middle panel), showing the typical CK2 feature of directly using GTP as phosphoryl donor. The purified tobacco kinase was also sensitive to heparin and strongly stimulated by polylysine (Fig. 3A, lower panel).

Since TGBp1 phosphorylation is increased in PVX-infected plants as compared to non-infected plants, the possible stimulation of CK2 transcription by PVX infection was explored by Northern blot analysis (Fig. 3B). Total RNA was obtained from leaves of PVX-infected and non-infected plants and hybridised to a probe able to recognise all three CK2A genes of *N. tabacum*, which are over 90% identical at the nucleotide level (Matsushita et al., 2003). The accumulation of CK2A mRNA transcripts was similar in PVX-infected and non-infected plants, indicating that the increased phosphory-





**Fig. 4.** Native TBGp1 phosphorylation by a *N. tabacum* CK2-like activity. Upper panels: PVX-infected (A and B) and non-infected (C) plant extracts were incubated with [ $\gamma$ - $^{32}$ P]-ATP in the presence of 5 mM MgCl<sub>2</sub> alone (lanes 3, 7 and 11) or together with the additives indicated, and TBGp1 was immunoprecipitated from the extracts using polyclonal TBGp1 antiserum (A and C) or the control pre-immune antiserum (B). Reactions were resolved in 15% SDS-PAGE, electroblotted onto nitrocellulose membranes and autoradiographed. Lower panels: immunoblotting of the membranes with anti-TBGp1 antiserum. Asterisks indicate a protein inespecifically precipitated by the antibody. Molecular mass markers (61, 47.8, 37, 24.5 and 19.2 kDa) are indicated on the right side of the autoradiographs. Additives: 100  $\mu$ M ATP, 100  $\mu$ M GTP, 10  $\mu$ M polylysine.

lation of TBGp1 in PVX-infected extracts is not due to an increase in CK2 transcription.

### 3.3. Phosphorylation of native PVX TBGp1 by a CK2-like activity

Detection of phosphorylated TBGp1 protein in PVX-infected extracts is a difficult task due to the low abundance of this protein and the reversible nature of phosphorylation. To determine whether native TBGp1 synthesized during PVX infection is a substrate for cellular kinases, PVX-infected extracts were incubated with [ $\gamma$ - $^{32}$ P]-ATP with or without additives and subsequently immunoprecipitated with a specific anti-TBGp1 antiserum (Fig. 4A) or pre-immune antiserum as negative control (Fig. 4B). A phosphorylated band with the molecular mass of TBGp1 (24 kDa) was specifically precipitated from PVX-infected extracts, while no proteins were immunoprecipitated by the pre-immune antiserum (upper panels of Fig. 4A and 4B, compare lanes 3 and 7). This phosphorylated 24 kDa band was specifically recognized in Western blot by a anti-TBGp1 antiserum (Fig. 4A, lower panel). Since it has been reported that PVX capsid protein (CP) interacts with TBGp1 *in vitro* and is also targeted by cellular kinases (Atabekov et al., 2001), the same immunoprecipitated proteins were revealed with an anti-PVX CP antiserum, but no CP immunoreactivity was detected (data not shown). Thus, these results indicate that native TBGp1 is phosphorylated by a cellular kinase.

Apart from the specific 24 kDa-TBGp1 protein, the anti-TBGp1 antiserum also precipitated a non-phosphorylated 30 kDa band (asterisk in lower panel of Fig. 4A). This protein was also immunoprecipitated by the TBGp1 antiserum from non-infected plant extracts incubated with [ $\gamma$ - $^{32}$ P]-ATP (asterisk in lower panel of Fig. 4C), showing that the 30 kDa corresponds to an endogenous plant protein.

The phosphorylation of native TBGp1 was inhibited by incubation with cold ATP and cold GTP, and was stimulated by polylysine (Fig. 4A upper panel, lanes 2–4). Taken together, these results are globally consistent with the *in vitro* assays, confirming that the native form of TBGp1 can be phosphorylated by an endogenous tobacco kinase and reinforcing the involvement of a CK2-like kinase.

### 3.4. TBGp1 phosphorylation by recombinant *N. tabacum* CK2 $\alpha$ subunit

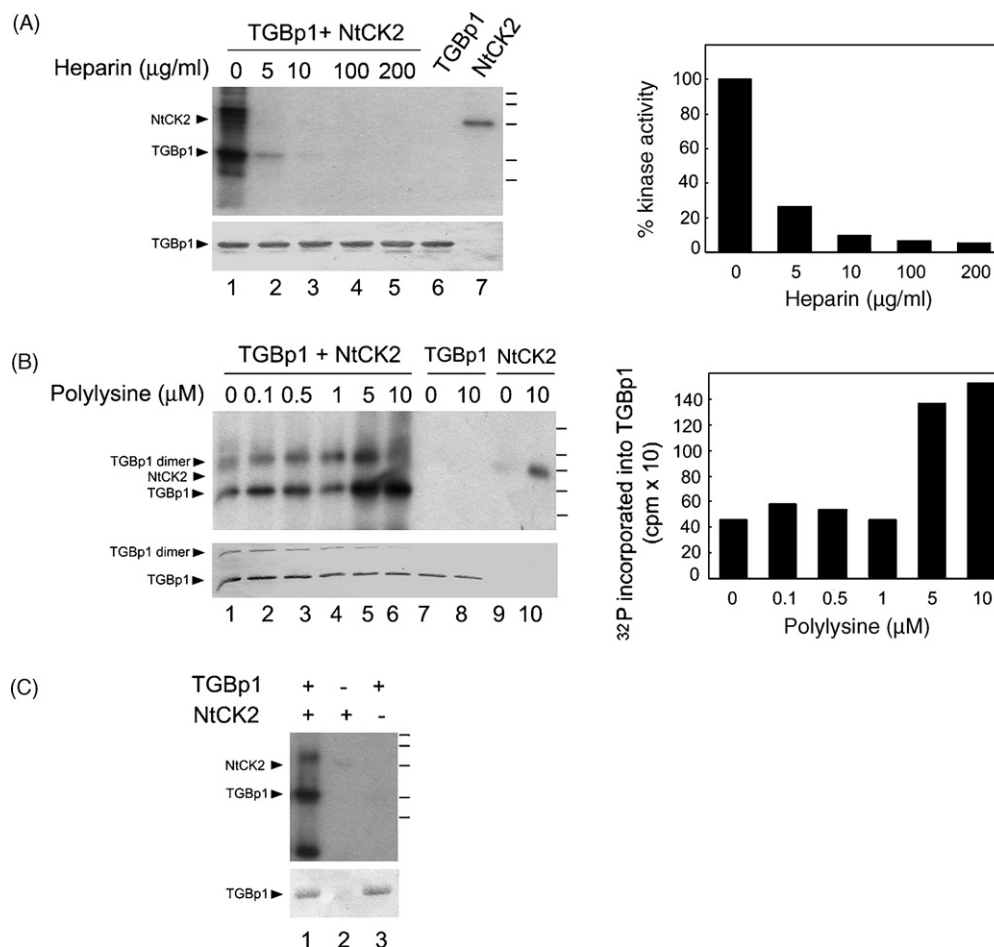
In order to employ a well-defined tobacco CK2 kinase, the  $\alpha$ -subunit of *N. tabacum* CK2 (NtCK2) was cloned, expressed in *E. coli* cells and purified to homogeneity. *In vitro* phosphorylation assays showed that recombinant TBGp1 was efficiently and specifically phosphorylated using NtCK2 as enzymatic source (Fig. 5A, lane 1 and Fig. 5B, lane 1). The *in vitro* phosphorylation of TBGp1 by NtCK2 was inhibited by about 70% with 5  $\mu$ g/ml heparin (Fig. 5A, compare lanes 1 to 2, and right panel).

Supplementation of the reaction mix with concentrations of up to 5  $\mu$ M polylysine led to a threefold increase in TBGp1 phosphorylation (Fig. 5B, compare lanes 1 and 5, and right panel). In this assay, phosphorylated TBGp1 appears as two bands recognised by the antiserum, and the slower-migrating band has a size compatible with a TBGp1 dimer. Higher polylysine amounts did not have further stimulatory effect on TBGp1 phosphorylation, but increased autophosphorylation of NtCK2 was observed at 10  $\mu$ M (Fig. 5B, lanes 9 and 10).

TBGp1 was also phosphorylated by the NtCK2  $\alpha$  subunit in the presence of [ $\gamma$ - $^{32}$ P]-GTP and Mn<sup>2+</sup> (Fig. 5C, lane 1). This provides direct evidence showing that tobacco CK2 is able to use both ATP and GTP as phosphoryl donors.

### 3.5. Ser-165 of PVX MP is phosphorylated by CK2

Sequences in PVX TBGp1 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.org/prosite/>). Four CK2 consensus sites were identified: 89-TTRE-92, 140-SQEE-143, 165-SEAE-168 and 185-TGLE-188. To characterize the sites of protein modification, PVX TBGp1 was phosphorylated with recombinant NtCK2  $\alpha$ -subunit and 50  $\mu$ M ATP and subjected to matrix-assisted laser desorption/ionization tandem time-of-flight spectrometry (MALDI-TOF/TOF). This assay revealed the presence of a single phosphopeptide of  $M_r$  1073.6, identified as 160-VIAIDSEAEK-169,



**Fig. 5.** *In vitro* TGBp1 phosphorylation by recombinant *N. tabacum* CK2  $\alpha$  subunit. (A) Left panel: phosphorylation assays of TGBp1 in the presence of [ $\gamma$ - $^{32}$ P]-ATP and increasing concentrations of heparin (lanes 1–5). Control reactions were made with TGBp1 alone (lane 6) and NtCK2 alone (lane 7). The lower panel shows a Western blot of the gel with an anti-TGBp1 antiserum. Right panel: percentage of TGBp1 phosphorylation in the presence of increasing concentrations of heparin relative to TGBp1 phosphorylation without heparin (100%). (B) Left panel: phosphorylation assays of TGBp1 in the presence of [ $\gamma$ - $^{32}$ P]-ATP and increasing concentrations of polylysine (lanes 1–6). Control reactions were performed with TGBp1 alone (lanes 7 and 8) and CK2 alone (lanes 9 and 10) in the presence of 0 and 10  $\mu$ M polylysine. The lower panel shows a Western blot of the gel with an anti-TGBp1 antiserum. Right panel: percentage of TGBp1 phosphorylation in the presence of increasing concentrations of polylysine. (C) TGBp1 phosphorylation in the presence of [ $\gamma$ - $^{32}$ P]-GTP and  $MnCl_2$  (lane 1). Control reactions were made with NtCK2 alone (lane 2) and TGBp1 alone (lane 3). Products of the reaction mixes were resolved in 15% SDS-PAGE and autoradiographed. The lower panel shows a Western blot of the gel with an anti-TGBp1 antiserum. Molecular mass markers (61, 47.8, 37, 24.5 and 19.2 kDa) are indicated on the right side of the autoradiographs.

thus indicating that Ser-165 is a site of PVX TGBp1 phosphorylation by NtCK2.

#### 4. Discussion

Using different experimental approaches, we have found that the TGBp1 of PVX is phosphorylated by a kinase present in tobacco plants. We obtained consistent evidence indicating that the kinase involved in TGBp1 phosphorylation belongs to the CK2 family. First, *in vitro* phosphorylation of TGBp1 by tobacco extracts is stimulated by polylysine, inhibited by heparin and able to use both ATP and GTP as phosphoryl donors. Second, an endogenous activity able to phosphorylate recombinant TGBp1 was partially purified by heparin-chromatography and its enzymatic properties also correspond to those of CK2. Third, direct phosphorylation of recombinant PVX TGBp1 was achieved using recombinant tobacco CK2  $\alpha$  subunit as enzymatic source. Fourth, the results obtained with recombinant TGBp1 were confirmed with the native protein, since we observed that native TGBp1 is phosphorylated by a plant kinase exhibiting similar properties to those of *N. tabacum* CK2 catalytic subunit. Since competition with cold GTP and stimulation by polylysine are

particularly good diagnostic tools for members of this family, we conclude that TGBp1 is most likely phosphorylated by CK2 during viral infection.

Viral infection increases the phosphorylation of TGBp1 by plant extracts, even though the amount of CK2  $\alpha$  subunit mRNA is not increased upon PVX infection, indicating that the kinase activity of existing CK2 may be increased. On the other hand, since the addition of heparin had a much greater effect on the phosphorylation of TGBp1 using extracts from non-infected plants than with extracts from infected plants and the same was true for the effect of adding GTP, it is possible that some other kinase apart from CK2 may be stimulated in infected plants that is not stimulated in non-infected plants.

CK2 is a ubiquitous, constitutively active Ser/Thr protein kinase present in all eukaryotic cells. In plants, CK2 is a heterogeneous complex composed of different catalytic  $\alpha$  and regulatory  $\beta$  subunits, although a monomeric form has also been detected (Dobrowolska et al., 1992; Riera et al., 2001). CK2 is mostly located in nuclei and cytoplasm, but it is also associated with specific structures or organelles, including the plasma membrane, Golgi and endoplasmic reticulum (reviewed in Litchfield, 2003; Salinas et al.,

2006). Our results indicate that PVX TGBp1 is phosphorylated by a cellular CK2 kinase present in the soluble fraction of tobacco plant extracts. Since this fraction contains the cytoplasm and endoplasmic reticulum and plasma membrane remainings, this distribution is compatible with the localization of TGBp1, which was previously shown to form small aggregates throughout the cytoplasm (Howard et al., 2004). Interestingly, CK2, along with other kinases, has been shown to phosphorylate the PVX capsid protein (CP; Atabekov et al., 2001). CP phosphorylation promotes the translation of the encapsidated viral RNA *in vitro*, suggesting that the translational activity of PVX in primary inoculated cells may be regulated by this process (Atabekov et al., 2001). Thus, CK2 might be involved in regulating several aspects of PVX infection via its effects on viral CP and MP.

There are few reports on the phosphorylation of plant viral MPs, and most studies have been performed with two related tobamoviruses, TMV and ToMV (Citovsky et al., 1993; Kawakami et al., 1999; Waigmann et al., 2000; Trutnyeva et al., 2005). TMV MP is phosphorylated by a kinase present in cell wall-enriched extracts (Waigmann et al., 2000), that is associated with plasmodesmata and belongs to the casein kinase 1 family (Lee et al., 2005). Experiments with phosphorylation mutants have shown that TMV MP phosphorylation negatively regulates viral transport through plasmodesmata in a host-dependent manner (Waigmann et al., 2000). A more detailed analysis showed that TMV-MP phosphorylation at a single amino acid residue actually promotes viral movement, while further phosphorylation impairs viral movement (Trutnyeva et al., 2005). The MP of ToMV, on the other hand, has been shown to be *in vitro* phosphorylated by CK2 (Matsushita et al., 2003) and to interact with, and be phosphorylated by, a novel tobacco kinase called R10 kinase (NtRIO) (Yoshioka et al., 2004). CK2 is able to phosphorylate NtRIO *in vitro*, and this modification inhibits the interaction between NtRIO and ToMV MP (Yoshioka et al., 2004).

The possible roles of TGBp1 phosphorylation on PVX infection remain unclear. Recent studies using PVX and related potexviruses have shown that TGBp1 is a multifunctional protein (Verchot-Lubicz, 2005; Verchot-Lubicz et al., 2007). Functions assigned to PVX TGBp1 include increasing plasmodesmata exclusion limit (Angell et al., 1996; Lough et al., 1998; Howard et al., 2004), suppression of post-transcriptional gene silencing (Voinnet et al., 2000), promoting transport of viral molecules through plasmodesmata (Lough et al., 1998, 2000), and promotion of the translation of viral RNA (Atabekov et al., 2000; Rodionova et al., 2003). The various functions attributed to TGBp1 are presumably dependent on a succession of interactions between TGBp1 and several viral and host proteins and ribonucleoprotein complexes, but the molecular mechanisms involved in these interactions are unknown. In this context, successive TGBp1 phosphorylations by CK2 or other cellular kinases might be regulating TGBp1 interactions with other viral/host proteins and its traffic through different cellular compartments. Further studies employing full-length cDNA copies of the PVX genome carrying specific mutations at candidate phosphorylation sites of TGBp1 will help clarify the function(s) of MP phosphorylation on PVX infection.

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