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Phosphorylation of TGB1 by protein kinase CK2 promotes barley stripe mosaic virus movement in monocots and dicots

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Abstract

The barley stripe mosaic virus (BSMV) triple gene block 1 (TGB1) protein is required for virus cell-to-cell movement. However, little information is available about how these activities are regulated by post-translational modifications. In this study, we showed that the BSMV Xinjiang strain TGB1 (XJ TGB1) is phosphorylated in vivo and in vitro by protein kinase CK2 from barley and Nicotiana benthamiana. Liquid chromatography tandem mass spectrometry analysis and in vitro phosphorylation assays demonstrated that Thr-401 is the major phosphorylation site of the XJ TGB1 protein, and suggested that a Thr-395 kinase docking site supports Thr-401 phosphorylation. Substitution of Thr-395 with alanine (T395A) only moderately impaired virus cell-to-cell movement and systemic infection. In contrast, the Thr-401 alanine (T401A) virus mutant was unable to systemically infect N. benthamiana but had only minor effects in monocot hosts. Substitution of Thr-395 or Thr-401 with aspartic acid interfered with monocot and dicot cell-to-cell movement and the plants failed to develop systemic infections. However, virus derivatives with single glutamic acid substitutions at Thr-395 and Thr-401 developed nearly normal systemic infections in the monocot hosts but were unable to infect N. benthamiana systemically, and none of the double mutants was able to infect dicot and monocot hosts. The mutant XJ TGB1 T395A/T401A weakened in vitro interactions between XJ TGB1 and XJ TGB3 proteins but had little effect on XJ TGB1 RNA-binding ability. Taken together, our results support a critical role of CK2 phosphorylation in the movement of BSMV in monocots and dicots, and provide new insights into the roles of phosphorylation in TGB protein functions.

Key words: Barley stripe mosaic virus, triple gene block 1 (TGB1) protein, phosphorylation, protein kinase CK2, promotion, viral movement.

Introduction

Barley stripe mosaic virus is the type species of the genus Hordeivirus. Barley stripe mosaic virus (BSMV) infects barley, wheat, and oats under natural conditions, and numerous other monocots and dicots by artificial inoculation (Bragg et al., 2008; Jackson et al., 2009). BSMV has recently been isolated from 750-year-old barley grains found near the Nile...
River (Smith et al., 2014), and consists of a large number of strains that were early subjects of phenotypic and host-range studies (McKinney and Greeley, 1965). BSMV has a positive-sense tripartite RNA genome (RNAα, -β, and -γ) that encodes seven major proteins (Bragg et al., 2008; Jackson et al., 2009). RNAα directs synthesis of the α protein, which functions as the methyltransferase/helicase subunit of the replicase [RNA-dependent RNA polymerase (RdRp)]. RNAβ encodes [α coat protein (CP)], and an overlapping triple gene block (TGB) sequence encoding three major movement proteins (TGB1, TGB2, and TGB3) that are expressed from two subgenomic RNAs. RNAγ serves as the mRNA for the γa RdRp polymerase subunit, and the γb protein, which functions as a suppressor of RNA silencing and a modulator of host defences (Jackson et al., 2009).

The BSMV TGB1 protein is a multifunctional 58 kDa protein with RNA-binding, RNA helicase, and ATPase activities (Donald et al., 1997; Verchot-Lubicz et al., 2010). The C-terminal region contains seven conserved helicase motifs, and mutations within one or more of these motifs have been shown to be involved in enzymatic and movement functions and RNA-binding activities (Lawrence and Jackson, 2001; Lim et al., 2008). TGB2 and TGB3 are trans-membrane proteins that are integrated in the endoplasmic reticulum bilayer (Morozov and Solovyev, 2003). Cell-to-cell movement of BSMV does not require the CP, and this property has permitted isolation of nucleoprotein complexes composed of the TGB1 protein and viral genomic and subgenomic RNAs (Lim et al., 2008). The TGB1 protein interacts directly with the TGB3 protein and indirectly with the TGB2 protein to form heterologous complexes required for co-localization of the TGB proteins at the plasmodesmata (PD) and BSMV cell-to-cell movement (Lawrence and Jackson, 2001; Lim et al., 2008; Jackson et al., 2009; Lim et al., 2009). Our recent results have shown that TGB1 proteins function in eliciting resistance to BSMV strains that are unable to infect Brachypodium distachyon inbred lines containing the Bsr1 resistance gene. In the case of the North Dakota 18 (ND18) strain TGB1 protein, amino acid residues at positions 390 and 392 are critical for TGB1 protein genetic interactions with Bsr1 and for inducing resistance responses (Cui et al., 2012; Lee et al., 2012).

Although the studies mentioned above provide valuable insights into BSMV cell-to-cell movement processes, little information is available about post-translational biochemical events, such as phosphorylation, that may function in regulating intercellular macromolecular trafficking. Movement protein (MP) phosphorylation was first demonstrated to be important for virus cell-to-cell movement during investigations with the tobacco mosaic virus (TMV) 30 kDa MP, and this study provided important approaches for subsequent MP analyses (Citovsky et al., 1993). Several proteins may function in TMV movement, because a cell-wall-associated kinase (Citovsky et al., 1993), a PD-associated protein kinase (PAPK1) (Lee et al., 2005), an endoplasmic reticulum-associated kinase (Karger et al., 2003), and protein kinase CK2 (formerly known as casein kinase II; Ivanov et al., 2003) have been shown to be involved in in vitro and in vivo phosphorylation of the 30 kDa protein (Citovsky et al., 1993). Moreover, mimicking MP phosphorylation by negatively charged amino acids inhibited MP transport through PD and delayed TMV and potyvirus spread in Nicotiana tabacum (Waignmann et al., 2000; Karger et al., 2003). More recently, phosphorylation of the MPs of other viruses, such as tomato mosaic virus (Kawakami et al., 2003; Matsushita et al., 2003), potato leafroll virus (Link et al., 2011), Abutilon mosaic virus (Kleinow et al., 2009), brome mosaic virus (Akamatsu et al., 2007), apple chlorotic leaf spot virus (Sato et al., 1995), and cucumber mosaic virus (Matsushita et al., 2002), has been shown to either enhance or inhibit virus movement during infection.

Although little information is available about the roles of phosphorylation in the movement processes of TGB MPs, potato virus X TGB1 protein is efficiently phosphorylated by N. tabacum protein kinase CK2 (Modena et al., 2008). Furthermore, tyrosine phosphorylation regulates the functions of potato mop-top virus TGB3 protein because substitution of tyrosine residues in two phosphorylation domains enhances interactions between the TGB3 and TGB2 proteins and inhibits virus cell-to-cell movement (Samuilova et al., 2013). In addition, the N-terminal half of the TGB1 protein of Poa semilatent virus (PSLV), a hordeivirus closely related to BSMV, has been reported to be phosphorylated in vitro by casein kinase 1 (CK1), protein kinase A (PKA), and protein kinase C (PKC)-like kinases present in N. benthamiana cell-wall fractions. In the case of PSLV, phosphorylation of an internal domain decreases RNA-binding activity and homologous protein–protein interactions, but experiments to determine whether these activities affect movement have not been conducted (Makarov et al., 2012). Here, we present the first evidence that BSMV TGB1 protein is phosphorylated in vitro and in vivo by the host protein kinase CK2. Our biochemical and molecular approaches demonstrated that Thr-401 in the TGB1 C-terminal region is a major phosphorylation site of the TGB1 protein, and that the Thr-395 residue serves as a CK2 docking domain. Mutational analyses of these residues indicate that a phosphorylation-dependent mechanism is involved in BSMV local and systemic infections in monocots and dicots.

Materials and methods

Plant growth conditions

* N. benthamiana* plants were grown in a climate chamber at 23 to 25 °C with a 14/10 h light (~75 mmol m⁻² s⁻¹)/dark photoperiod as described previously (Yuan et al., 2011). Barley (Yangpi 8), wheat (Yangmai 11), and *B. distachyon* Bd21 plants were grown in a greenhouse until the two-leaf stage, and then inoculated and transferred to a climate chamber at the same temperature and light regimen as above until evaluated at 5 to 12 d post-inoculation (dpi).

Construction of infectious clones of BSMV Xinjiang

Genomic (g) RNAs of *α*BSMV strain were extracted from purified virus with Trizol (Life Technologies) and used to prime reverse transcription of the gRNAs with primer BS32 as described previously (Yuan et al., 2011; Lee et al., 2012). BSMV α, β, and γ-cDNAs were amplified with the primer pairs XJ-1/BS32, XJ-2/BS32, and
XJ-3/BS32, respectively (Supplementary Table S1, available at JXB online), and inserted into the pMD20-T vector (Takara) to generate pT7-αXJ, pT7-βXJ, and pT7-γXJ. Site-specific mutagenesis was carried out with a QuikChange Site-Directed Mutagenesis kit (Agilent Technologies) to make alanine (A), aspartic acid (D), and glutamic acid (E) substitutions for XJ TGB1 protein residues 395 and 401 in pT7-βXJ with the corresponding primer pairs (Supplementary Table S2, available at JXB online). These clones and all those described below were verified by DNA sequencing (Tsingke Biotech, Beijing).

To engineer XJ BSMV derivatives for agroinfiltration, full-length cDNAs were amplified from pT7-αXJ, pT7-βXJ (or the site-specific pT7-βXJ1 TGB1 G3P TGB1 viruses), and pT7-γXJ clones with the primer pairs CH10/BS26, CH11/BS26, and CH12/BS26, respectively (Supplementary Table S1). The cDNAs then were inserted between the StuI and BamHI sites of pCass4-Rz (Annamalai and Rao, 2005), and the resulting clones and site-specific mutants with the 395 and 401 residue substitutions were designated pCa-αXJ, pCa-βXJ, pCa-γXJ, and pCa-βXJ2 TGB1 mutants.

Mechanical inoculation of in vitro transcripts and agroinfiltration of BSMV derivatives

The pT7-αXJ, pT7-βXJ, and pT7-γXJ plasmids were linearized with SpeI or BamHI and used as templates for T7 RNA polymerase (Promega) in vitro transcription of capped infectious RNAs (Petty et al., 1989). The α, β, and γ in vitro transcripts were mixed in equal amounts with FES inoculation buffer (0.1 M glycine, 0.06 M potassium phosphate, 1% sodium pyrophosphate decahydrate, 1% bentonite, 1% cetine, pH 8.5) and used immediately for inoculation of 7- to 10-d-old barley, wheat, and B. distachyon Bd21. Plasmids pCa-αXJ, pCa-βXJ, and pCa-γXJ were maintained in Agrobacterium tumefaciens strain EHA105 and infiltrated into the lower side of N. benthamiana leaves as described previously (Yuan et al., 2011).

Immunoprecipitation

Immunoprecipitation assays were carried out with minor modifications of a published protocol (Rubio et al., 2005). N. benthamiana leaf sections were harvested at 5 d after agroinfiltration and proteins were extracted in 2 vol of GTEN buffer [10% (v/v) glycerol, 25 mM Tris/HCl (pH 7.5), 1 mM EDTA, 150 mM NaCl, 10 mM dithiothreitol, 2% (w/v) polyvinylpolypyrrolidone, 1% Protease Inhibitor Cocktail (Roche)]. Extracted complexes were stirred for 10 min at 4°C and then mixed with protein G–agarose (Millipore) at 1:500 dilutions.

Soluble cytoplasmic protein extracts of healthy N. benthamiana leaves were used for in vitro kinase assays according to the protocol described by Hung et al. (2014) and Vijayapalani et al. (2012). Phosphorylation reactions were performed with the N. benthamiana soluble protein extracts or with purified recombinant NbCK2α and HvCK2α subunits. Assays were performed with 1 µg of N. benthamiana soluble protein extracts, or with 0.1 µg of recombinant CK2α, and 1 µg of purified XJ TGB1 protein or its mutants in a final volume of 10 µl of 25 mM Tris/HCl (pH 7.4), 10 mM MgCl2, and 1 µl [γ-32P]ATP or GTP (10 µCi, ~300 Ci mmol⁻¹; Perkin Elmer). Selected reactions were carried out in the presence or absence of heparin, and various concentrations of unlabelled ATP or GTP. Negative controls contained no TGB1 protein or 500 and 1000 ng of bovine serum albumin. After incubation at 30 °C for 30 min, the reactions were terminated by addition of 2.5 µl of 5X SDS buffer, and the samples were subjected to 12.5% SDS-PAGE. The gels were dried with a Model 583 Gel Dryer (Bio-Rad) and phosphorylated proteins were visualized by autoradiography.

Mass spectrometry analysis

Phosphorylated XJ TGB1 and unphosphorylated XJ TGB1 proteins were digested with trypsin at 37 °C overnight. The digested peptides were then analysed by Q-Exactive liquid chromatography tandem mass spectrometry (LC-MS/MS) (Thermo Scientific) at the Mass Spectrometry Facility at China Agricultural University. The data were searched against the NCBI database using Mascot software with a 1% false discovery rate.

Fluorescence and confocal microscopy

Green fluorescent protein (GFP) or red fluorescent protein (RFP) fluorescence in epidermal cells of N. benthamiana was observed with an Olympus confocal FV1000 microscope. GFP and RFP were excited at 488 or 546 nm, respectively, with an argon laser. Images were then compared and analyzed with a fluorescence microscope (Carl Zeiss, Jena, Germany) and confocal microscope (Nikon, Tokyo, Japan) with the NIS-Elements software (Nikon).
were recorded with an Olympus camera and processed using an Olympus Fluoview version 3.0 Viewer. In addition, cell-to-cell movement assays in epidermal cells of barley and *N. benthamiana* were observed with a BX53+DP72 fluorescence microscope (Olympus) and images were manipulated with the cellSens Entry programs.

Electrophoretic mobility shift assays (EMSA)

RNAs for binding assays were transcribed *in vitro* in the presence of digoxigenin (DIG)–UTP (Roche) and the DIG-labelled transcripts were purified to remove the DNA templates (Wu et al., 2013). Phosphorylation reactions were performed in a final volume of 5 μl containing phosphorylation assay buffer and purified NbCK2α, and different amounts of recombinant XJ TGB1 protein as described above. Negative controls consisted of samples lacking recombinant XJ TGB1 protein or 500 and 1000ng of bovine serum albumin. EMSA binding comparisons were performed by adding increasing amounts of protein to 50ng of purified RNA in binding buffer [50mM Tris/ HCl (pH 7.5), 10mM MgCl₂, 1 mM EDTA] in a final volume of 20 μl. The binding reaction mixtures were incubated on ice for 30 min and subjected to electrophoresis on 1% (w/v) non-denaturing agarose gels in 0.5x TBE buffer. RNA–protein complexes were transferred to a Hybond N+ nylon membrane (GE Healthcare) via a pump suction filter, and the RNA was cross-linked to the membrane by two 60 s cycles at 0.12 J in a Bio-Link crosslinker (Vilber Lourmat). Mobility shifts of the DIG-labelled RNAs were detected with DIG– alkaline phosphatase Fab fragments (Roche), and the RNA was cross-linked to the membrane by two 60 s cycles at 0.12 J in a Bio-Link crosslinker (Vilber Lourmat). Mobility shifts of the DIG-labelled RNAs were detected with DIG– alkaline phosphatase Fab fragments (Roche), and the blots were developed with a 5-bromo-4-chloro-3-indolyl-phosphate/ nitro blue tetrazolium chloride substrate solution (Amresco).

GST pull-down

For co-expression of GST–XJ TGB3 with XJ TGB1–His or the XJ TGB1+HisΔ5+10–1 His fusion proteins, relevant plasmids were co-transformed into *E. coli* BL21(DE3). Cells were harvested by low-speed centrifugation and disrupted by vortexing in TB buffer [20mM Tris/HCl (pH 7.3), 500mM NaCl] in the presence of glass beads. The GST fusions and bound TGB proteins were purified by glutathione–Sepharose affinity chromatography and elution with glutathione (Pharmacia).

Results

Construction and sequence analysis of infectious clones of the BSMV Xinjiang strain

 Several BSMV field strains from China collected in our laboratory have broader host ranges than the more extensively studied XJ BSMV and Type BSMV (gly2 BSMV) strains. To evaluate the diversity of the more virulent BSMV strains (Lee et al., 2012), we constructed infectious clones of the XJ BSMV strain (Xie et al., 1981) under the bacteriophage T7 or double cauliflower mosaic virus 35S promoters (Petty et al., 1989; Yuan et al., 2011) (Fig. 1A). The infectivity of *in vitro* transcripts synthesized from linearized pT7–αXJ, pT7–βXJ, and pT7–γXJ plasmids was tested by mechanical inoculation to barley, wheat, and *B. distachyon* Bd21. Inoculated plants consistently developed chlorotic stripes and mosaic symptoms typical of BSMV infections on upper uninoculated leaves by 6–7 dpi (Fig. 1B) and the efficiency of infectivity in barley, wheat, and *B. distachyon* Bd21 was 70–80, 80–90, and 50–60% respectively (also see Supplementary Table S6). Agroinfiltration was used to initiate infections of *N. benthamiana* because only 10–30% of the plants became infected when using *in vitro* transcripts as inocula. *Agrobacterium* harbouring the plasmids pCa–αXJ, pCa–βXJ, and pCa–γXJ were infiltrated into the basal sides of the leaves. Newly emerging leaves developed mild mottling symptoms at 7–9 d after agroinfiltration (Fig. 1C), and the efficiency of infectivity was increased to approximate 90%. RT-PCR and Western blot analysis verified the infectivity of the XJ BSMV infectious clones in the monocot hosts (Fig. 1B) and in *N. benthamiana* (Fig. 1C).

For comparisons of XJ BSMV genomic variation with other published BSMV strains, the pT7–αXJ, pT7–βXJ and pT7–γXJ cDNAs were sequenced. The results indicated that XJ RNAα (GenBank accession no. KJ746471), XJ RNAβ (GenBank accession no. KJ746472), and XJ RNAγ (GenBank accession no. KJ746473) consisted of 3789, 3222, and 2793 nt, respectively, and shared nucleotide identities of 95.2–99.8% (Supplementary Table S3, available at JXB online), 94.1–99.2% (Supplementary Table S4, available at JXB online), and 87.9–98.8% (Supplementary Table S5, available at JXB online) with RNAs α, β, and γ of other BSMV strains. These results suggest that substantial diversity may exist among BSMV strains.

The XJ TGB1 protein is phosphorylated in vitro and in vivo

In order to explore phosphorylation *in vitro*, the full-length XJ TGB1 protein was expressed as a C-terminal His-tagged
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fusino protein and purified to near homogeneity by Ni-affinity chromatography (Fig. 2A). A soluble protein kinase is known to be present in tobacco species (Hung et al., 2014), and hence the purified \( x_j \)TGB1 protein was first assayed for phosphorylation using \( N. \) benthamiana protein extracts as a kinase source. The first control reaction containing [\( \gamma \-32P \)]ATP and cytoplasmic extracts without the \( x_j \)TGB1 protein resulted in no distinct labelled products (Fig. 2B, lane 1). The corresponding control with the \( x_j \)TGB1 protein and [\( \gamma \-32P \)]ATP alone suggested that the \( x_j \)TGB1 protein was not autophosphorylated in vitro (Fig. 2B, lane 2). In contrast, when both the cytoplasmic extracts and the \( x_j \)TGB1 protein were present, a radioactive phosphorylated product co-migrated with the \( x_j \)TGB1 protein (Fig. 2B, lane 3). These results provide evidence that the \( x_j \)TGB1 protein is phosphorylated in vitro with [\( \gamma \-32P \)]ATP by a soluble kinase in the \( N. \) benthamiana extracts. The vast majority of protein kinases use ATP as an exclusive phosphate donor, whereas CK2 can effectively use either ATP or GTP (Matsushita et al., 2000); hence we carried out phosphorylation comparisons with GTP to obtain clues about the identity of the kinase involved in \( x_j \)TGB1 phosphorylation. Autoradiography of the phosphorylated products revealed a single intense radio labelled band when either [\( \gamma \-32P \)]ATP or [\( \gamma \-32P \)]GTP was used as a phosphoryl donor (Fig. 2B, lanes 3 and 4), implying that \( x_j \)TGB1 is phosphorylated by a CK2-like kinase in \( N. \) benthamiana.

To determine whether the \( x_j \)TGB1 protein is phosphorylated in vivo, \( x_j \)BSMV-infiltrated \( N. \) benthamiana leaves were harvested, concentrated by immunoprecipitation, and subjected to Western blot analysis with an anti-phosphothreonine (\( \alpha \-pT \)) antibody. The \( \alpha \-pT \) results revealed a labelled protein with an electrophoretic mobility corresponding to the 58 kDa \( x_j \)TGB1 protein (Fig. 2C and D, top, lane 3). Similarized bands were not observed in mock-inoculated leaves, or leaves infiltrated with only pCa-\( \alpha \) and pCa-\( \gamma \) (Fig. 2C and D, top, lanes 1 and 2). Western blot analyses using the \( x_j \)TGB1 protein antibody confirmed that the \( \alpha \-pT \) reactivity protein co-migrated with \( x_j \)TGB1, and revealed a 45 kDa immunospecific band that we suspect is a degradation product (Fig. 2C and D, bottom, lane 3). Collectively, these experiments provide convincing evidence that the \( x_j \)TGB1 protein is phosphorylated by a soluble CK2-like kinase in vitro and in vivo.

CK2 is able to phosphorylate the \( x_j \)TGB1 protein

To predict potential \( x_j \)TGB1 protein phosphorylation kinases and sites, we analysed the \( x_j \)TGB1 protein sequence with the GPS 2.1 program tool (Xue et al., 2011) and the Scansite Motif Scanner online server (http://scansite.mit.edu) (Obenauer, 2003). The GPS 2.1 program was set at a HIGH threshold (false-positive rates of 2% for Ser/Thr kinases) and the Scansite was set at a high stringency level to predict potential phosphorylation targets in the \( x_j \)TGB1 protein. According to the conserved CK2 phosphorylation site motif [(S/T)-X-X-(D/E); Meggio and Pinna, 2003], the GPS 2.1 predictions suggested that three residues in the \( x_j \)TGB1 protein (Ser-69, Thr-395, and Thr-401) are potential CK2 phosphorylation sites, whereas the Scansite only indicated that the Thr-401 site is phosphorylated by CK2 (Supplementary Fig. S1, available at JXB online). For comparison with ND18, the \( x_j \)TGB1 amino acids Ser-69, Thr-395, and Thr-401 correspond to the ND18 TGB1 (\( x_\)TGB1) Ser-70, Thr-396, and Thr-402 residues, respectively.

Based on the GTP results and phosphorylation predictions, we suspected that a CK2-like kinase might be responsible for phosphorylation of the \( x_j \)TGB1 protein. Therefore, the CK2\( \alpha \) subunits from \( N. \) benthamiana (NbCK2\( \alpha \)) and barley (HvCK2\( \alpha \)) were cloned, and the C-terminal His-tagged fusion proteins were purified from \( E. \) coli cells (Fig. 3A). Subsequent in vitro phosphorylation assays revealed that the purified NbCK2\( \alpha \) and HvCK2\( \alpha \) proteins efficiently phosphorylated the recombinant \( x_j \)TGB1 protein in the presence of [\( \gamma \-32P \)]ATP (Fig. 3B). CK2 is highly sensitive to heparin inhibition (Matsushita et al., 2003), and our results confirmed that the
levels of phosphorylation were reduced proportionally with increasing heparin concentrations (Fig. 3C). To further test the kinase specificity, we used NbCK2α to compare XJ TGB1 and TMV P30 MP (Ivanov et al., 2003) protein phosphorylation in the presence of [γ-32P]ATP and [γ-32P]GTP (Fig. 3D). In both cases, the NbCK2α phosphorylation assays resulted in the presence of highly intense bands that co-migrated with the XJ TGB1 and P30 proteins, and the kinase exhibited similar activities in the presence of both ATP and GTP (Fig. 3D, lanes 3 and 4, and 6 and 7). Moreover, the absence of radioactive bands in reactions lacking the NbCK2α proteins confirmed that the 58 kDa XJ TGB1 and the P30 proteins are not autophosphorylated and the lack of radioactivity in the reactions without substrate proteins also indicated that the NbCK2α protein is not self-phosphorylated (Fig. 3D, lanes 2 and 5). In addition, tests were carried out in the presence of...
Mn²⁺, Mg²⁺, and Ca²⁺ to assess the cation specificity of CK2 phosphorylation (Niefind et al., 1999). These results revealed that NbCK2α exhibited similar phosphorylation intensities for αTGB1 and P30 in the presence of either Mn²⁺ or Mg²⁺ (Fig. 3E, lanes 2 and 3, and 7 and 8), and that ³²P incorporation was negligible in reactions containing Ca²⁺ (Fig. 3E, lanes 4 and 9). All of the in vitro phosphorylation data with the TGB1 protein were consistent with several published biochemical properties of CK2 (Ivanov et al., 2003; Hung et al., 2014).

Furthermore, to explore the potential co-localization of the αTGB1 protein and NbCK2α in planta, we conducted co-expression experiments with GFP:αTGB1 and DsRed:NbCK2α fusion proteins in N. benthamiana cells. Confocal microscopy revealed that GFP:αTGB1 was distributed in both the cytoplasm and nucleus, whereas DsRed:NbCK2α was present primarily in the nucleus. The co-localization of the two proteins indicated that the αTGB1 protein and NbCK2α interact in both the nucleus and cytoplasm in some manner (Fig. 3F, overlay channel). Taken together, the presented data demonstrate that αTGB1 is phosphorylated by CK2 in vitro and in planta.

Thr-401 is the major αTGB1 protein site for CK2 phosphorylation

To identify the phosphorylation sites of CK2, the purified αTGB1 protein was phosphorylated by NbCK2α in vitro with unlabelled ATP, and the gel-purified phosphorylated and unphosphorylated αTGB1 proteins were separated by PAGE and digested with trypsin. The trypsin digestion products were analysed by Q-Exactive LC-MS/MS. The analysis showed that 87.9% of the TGB1 protein amino acid sequence was covered, and revealed that the phosphorylated and unphosphorylated proteins differed in a unique monophosphorylated peptide (390GETDETKNIAFTVDTR416) with a 2103.9362 m/z peak corresponding to a neutral precursor ion lacking phosphoric acid (97.9769 Da). Based on the observed masses of the phosphorylated and unphosphorylated y₁₆ fragment ions (Fig. 4A), we conclude that the phosphorylated αTGB1 residue is located at Thr-401.

To verify the GPS 2.1 and Scanner predictions (Supplementary Fig. S1) and LC-MS/MS analysis of αTGB1 protein, we replaced one or both of the Thr-395 and Thr-401 residues with alanine residues to produce αTGB1Δ395A, αTGB1Δ401A, and αTGB1Δ395AT401A phosphorylation-deficient mutants. To mimic the phosphorylation state of the αTGB1 protein, Thr-395 and Thr-401 residues were substituted with aspartic acid (D) or glutamic acid (E) residues to produce the αTGB1Δ395D, αTGB1Δ395E, αTGB1Δ401D, and αTGB1Δ401E mutants. In vitro phosphorylation comparisons of the wild-type (wt) αTGB1 protein, and the Thr-395 and Thr-401 mutants were performed with HvCK2α and NbCK2α, respectively. Compared with the wt αTGB1 protein (Fig. 4B, lane 1), the phosphorylation level of the αTGB1Δ395A mutant protein was reduced partially (Fig. 4B, lane 2). However, both the αTGB1Δ395D and the αTGB1Δ395E mutant proteins incorporated slightly larger amounts of ³²P label than the wt αTGB1 protein (Fig. 4B, compare lane 1 with lanes 3 and 4), suggesting that the positive charges imparted by the aspartic acid and glutamic acid residues increased the kinase efficiency. As anticipated, the phosphorylation intensities of the αTGB1Δ395A (Fig. 4B, lane 5) and αTGB1Δ401E (Fig. 4B, lane 7) proteins only showed faint shadows. We also observed similar reductions during incorporation into the αTGB1Δ395D protein (Fig. 4B, lane 6). We believe that Thr-395 may have been phosphorylated, and this is supported by negligible incorporation into the three double mutants (Fig. 4B, lane 8; Fig. 4C, lanes 1–3). Thus, our interpretation of these results is that Thr-401 is a major phosphorylation site and that Thr-395 is a minor phosphorylation target.

Based on the data above and previous analyses of 308 CK2 phosphorylation sites for other proteins (Meggio and Pinna, 2003), we propose that Thr-395 is a docking site for CK2 because phosphorylation of this residue enhanced kinase activity at Thr-401. To evaluate this hypothesis, we used a double mutant, αTGB1Δ395A/T401A, for phosphorylation. The results revealed extremely low, if any, ³²P incorporation into other TGB1 residues (Fig. 4B, lane 8; Fig. 4C, lane 3). To determine whether there was ‘off-site’ targeting of other residues within αTGB1 at the proposed Thr-395 docking site, we engineered the αTGB1Δ395A/T401A and αTGB1Δ401A/T401A double mutants, which could not be phosphorylated at Thr-401, and both double mutants had negligible levels of ³²P incorporation (Fig. 4C, lanes 1 and 2). In summary, these results support the hypothesis that Thr-395 functions as a docking residue and that Thr-401 is the major phosphorylation site within the kinase motif of the αTGB1 protein.

Mutations of the αTGB1 protein phosphorylation site affect BSMV local and systemic infections of dicots and monocots

To determine whether the αTGB1 mutants affected systemic movement in N. benthamiana, leaves were infiltrated with Agrobacterium harbouring the pCa-αX1, wt pCa-βX1, or individual TGB1 mutant derivatives, and the pCa-γX1 clones. Three independent experiments revealed that only wt αTGB1 and the βX1-αTGB1Δ395A mutant, which exhibited slightly lower phosphorylation levels than the wt αTGB1 protein, were able to establish systemic infections at 10 dpi (Fig. 5A, lanes 2 and 3). None of the remaining infiltrations containing single or double αTGB1 mutants developed mosaic symptoms or invaded the upper uninoculated leaves as assessed by the absence of CP accumulation (Fig. 5A, lanes 4–11).

For systemic infectivity on monocots, barley and wheat leaves were co-inoculated with in vitro transcripts of pT7-αX1, pT7-βX1 (wt or mutant derivatives), and pT7-γX1. Visual observations, ELISA and RT-PCR analyses demonstrated that the barley and wheat plants had similar systemic infections on upper uninoculated leaves at 14 dpi after wt and mutant αTGB1 inoculations (Fig. 5B, C). In the case of βX1-TGB1Δ395A, a milder infection phenotype was noted on the systemically infected cereal leaves, compared with those of the wt βX1 infections (Fig. 5B, C, lanes 2 and 3), but the aspartic acid mutant βX1ΔTGB1Δ395D was unable to infect the plants.
systemically (Fig. 5B, C, lane 4). In contrast, when inoculated with the βXj-TGB1 T395E mutant, which more effectively mimics phosphorylation of threonine residues, the cereal plants exhibited CP accumulation levels that were similar to wt BSMV (Fig. 5B, C, lane 5). Moreover, the βXj-TGB1 T401E mutant elicited systemic infections, albeit with slightly lower CP levels (Fig. 5B, C, lane 8), but the levels were higher than those with βXj-TGB1 T401A (Supplementary Table S6, available at JXB online). However, the βXj-TGB1 T395A/T401A, βXj-TGB1 T395D/T401A, and βXj-TGB1 T395E/T401A double mutants were unable to infect either barley or wheat (Fig. 5B, lanes 9–11).

Taken together, these results demonstrated that the XjTGB1 phosphorylation site mutations generally reduced the infection efficiencies in dicots and monocots, and that the mutants had more dramatically compromised systemic movement phenotypes in N. benthamiana. To summarize, the T395A, T395E, T401A, and T401E mutants exhibited systemic movement in

![Fig. 4.](image-url)
Phosphorylation of BSMV TGB1 promotes viral movement

barley and wheat, whereas only the T395A mutant is able to establish systemic infections in *N. benthamiana*.

We next conducted experiments to evaluate the cell-to-cell movement profiles of the Thr-395 and Thr-401 mutants in *N. benthamiana* and barley. For this purpose, *N. benthamiana* leaves were infiltrated with *Agrobacterium* containing pCa-αXJ, pCa-βXJ, and pCa-γXJ, or its phosphorylation site mutants. Upper uninfiltrated leaf tissues were harvested and photographed at 10 dpi (top). CP ELISA (middle) and RNA γ RT-PCR amplification (bottom) were monitored to estimate the infectivity levels. (B, C) Systemic symptoms appearing in barley (B) and wheat (C) after inoculation with pT7-αXJ and pT7-γXJ in vitro transcripts mixed with pT7-βXJ and various phosphorylation mutant transcripts. Leaves were photographed at 14 dpi (top) and all experiments were repeated three times.

(This figure is available in colour at *JXB* online.)
RNA\textsubscript{\textgamma XJ\textbeta} transcripts. Epidermal cells of the leaves were observed by confocal microscopy at 3 dpi and compared with inoculated controls lacking the RNA\textbeta.

The localized movement in infiltrated *N. benthamiana* leaves generally reflected the systemic infection phenotypes elicited by the mutants (Fig. 6A). In *N. benthamiana*, the wt \( \beta_{XJ} \) and \( \beta_{XJ}\text{\textbeta T395A} \) mutant both exhibited cell-to-cell movement encompassing several cells at 3 dpi (Fig. 6A), as expected due to their ability to elicit systemic infections. The remaining mutants usually developed fluorescence in a single cell or rarely in two to three adjacent cells (Fig. 6A). Hence, the localized movements of the mutants correlated reasonably well with their systemic movement patterns in *N. benthamiana*. In barley leaves, most of the fluorescence at 3 dpi appeared in mesophyll cells, but in this case, the virus had to traverse only a limited number of mesophyll cells to reach the vascular elements for systemic spread, whereas movement through a larger number of cells was required to reach the dicot vasculature. The other mutants, \( \beta_{XJ}\text{\textbeta T395D} \), \( \beta_{XJ}\text{\textbeta T401D} \), and \( \beta_{XJ}\text{\textbeta T395A/T401A} \), exhibited more limited cell-to-cell movement compared with \( \beta_{XJ}\text{\textbeta T395E} \), \( \beta_{XJ}\text{\textbeta T395A} \), and \( \beta_{XJ}\text{\textbeta T401A} \), but could spread to a few adjacent cells. However, these three mutants were unable to invade the upper cereal leaves. Hence, these results demonstrate that phosphorylation activities at Thr-395 and Thr-401 differentially affect systemic movement in monocot versus dicot hosts and suggest that at least some of the host-specific results may be a consequence of the vasculature architecture of these hosts.

**Phosphorylation promotes virus infection of \( \chi_{BSMV} \) by enhancing TGB1 and TGB3 protein interactions**

Previous studies have shown that BSMV spreads from cell to cell through the co-ordinated actions of TGB proteins, which co-localize at the cell wall in close association with PD, during cell-to-cell movement in monocots and dicots (Lim et al., 2008). Our results shown above demonstrated that interference with CK2 phosphorylation at the \( \chi_{XJ}\text{TGB1} \) Thr-395 and Thr-401 sites affected \( \chi_{BSMV} \) local and systemic movement. TGB1 is a multifunctional protein that engages in homologous interactions and formation of a ribonucleoprotein complex containing viral genomic and subgenomic RNAs (Lawrence and Jackson, 2001). Therefore, we used three approaches to identify \( \chi_{XJ}\text{TGB1} \) protein functions affected by CK2 phosphorylation.

To determine whether the RNA-binding affinity of the \( \chi_{XJ}\text{TGB1} \) protein changed upon phosphorylation, we first used purified wt \( \chi_{XJ}\text{TGB1} \) and the double mutant \( \chi_{XJ}\text{TGB1\textbeta T395A/T401A} \) proteins in EMSA with DIG-labelled RNA transcripts (Fig. 7A). The results clearly showed that both proteins bound almost all of the available RNA at 250 ng, indicating that the mutant protein did not affect RNA-binding activities (Fig. 7A, panels 1 and 2). Next, to determine phosphorylation effects directly, the \( \chi_{XJ}\text{TGB1} \) protein was incubated in phosphorylation assay buffer containing unlabelled ATP, with and without the addition of purified NbCK2\( \alpha \), and EMSA assays were performed to compare the abilities of the non-phosphorylated and phosphorylated \( \chi_{XJ}\text{TGB1} \) proteins to bind the labelled RNA transcripts (Fig. 7A, panels 3 and 4). In addition, the RNA-binding activities of the phosphorylated wt \( \chi_{XJ}\text{TGB1} \) and mutant \( \chi_{XJ}\text{TGB1\textbeta T395A/T401A} \) proteins were compared, but the \( \chi_{XJ}\text{TGB1\textbeta T395A/T401A} \) protein was found to retain almost the same level of RNA binding as the wt \( \chi_{XJ}\text{TGB1} \) protein (Fig. 7A, panels 5 and 6). Hence, phosphorylation appeared to have little, if any, effect on the RNA-binding activities of the \( \chi_{XJ}\text{TGB1} \) protein.

To evaluate the possible role of phosphorylation in heterologous interactions of \( \chi_{XJ}\text{TGB1} \) and \( \chi_{XJ}\text{TGB3} \) proteins, experiments were carried out with His-tagged \( \chi_{XJ}\text{TGB1} \) and its mutant \( \chi_{XJ}\text{TGB1\textbeta T395A/T401A} \) fusion proteins in co-expressions with the GST:\( \chi_{XJ}\text{TGB3} \) protein in *E. coli* BL21 cells.

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**Fig. 6.** Effects of \( \chi_{XJ}\text{TGB1} \) protein phosphorylation on \( \chi_{BSMV} \) cell-to-cell movement in *N. benthamiana* and barley. (A) Fluorescence in *N. benthamiana* leaves at 3 dpi with an *Agrobacterium* mixture of pCa-\( \chi_{\textalpha XJ} \), pCa-\( \chi_{\textgamma XJ GFP} \), and pCa-\( \beta_{XJ} \) or the pCa-\( \beta_{XJ} \) mutant derivatives. The total bacterial concentrations for infiltration were OD\textsubscript{600} of 0.08. (B) Fluorescence in barley leaves at 3 dpi with in vitro transcripts of RNA\textalpha and RNA\textgamma GFF plus wt \( \chi_{XJ}\text{RNA}\beta \) or the \( \chi_{XJ}\text{RNA}\beta \) phosphorylation site mutant derivatives. Bars represent 500 \( \mu \)m. (This figure is available in colour at JXB online.)
Phosphorylation of BSMV TGB1 promotes viral movement

XJ-TGB1 and XJ-TGB1_{T395A/T401A} proteins were retained to some extent on the affinity columns by the GST:XJ-TGB3 protein. The concentrations of the TGB proteins were similar in the experiments, but the XJ-TGB1_{T395A/T401A} protein had approximate 40% TGB3 protein-binding efficiency compared with the wt XJ-TGB1 protein. The illustrated binding result is typical of three independent experiments. (C) Co-localization of TGB proteins. Confocal laser-scanning microscopy observation of N. benthamiana leaf epidermal cells co-infiltrated with mixtures of Agrobacterium harbouring GFP:XJ-TGB1 or the GFP:XJ-TGB1_{T395A/T401A} mutant derivatives and the pGD-TGB2 and RFP-TGB3 plasmids. Bars, 50 μm. (This figure is available in colour at JXB online.)

 approximate 40% less effectively than the XJ-TGB1 protein. These results thus suggested that the mutant XJ-TGB1_{T395A/T401A} may weaken TGB1:TGB3 protein interactions and result in impaired cell-to-cell movement functions of XJ-BSMV.
In additional attempts to ascertain whether the compromised TGB1:TGB3 protein interactions or CK2 phosphorylation affected localization of TGB proteins in plant cells, co-localization assays were performed by transient expression of the three TGB proteins via agroinfiltration in *N. benthamiana*, and GFP and RFP localizations were evaluated at 2 dpi by confocal laser-scanning microscopy. The results revealed that TGB2 and TGB3, the $\chi_T$TGB1$^{T395A/T401A}$ protein, and the wt $\chi_T$TGB1 protein had similar TGB localization patterns (Fig. 7C). Taken together, we conclude that phosphorylation promotes $\chi_T$BSMV infection by enhancing the interactions between the $\chi_T$TGB1 and $\chi_T$TGB3 proteins, although this effect was not sufficient to substantially alter the TGB localization patterns visible by confocal microscopy.

**Discussion**

Reversible phosphorylation and dephosphorylation of proteins have regulatory roles in a wide range of cellular processes, including cell signalling transduction (Moreno-Romero et al., 2011), protein subcellular localization (Nardozzi et al., 2010), and protein–protein (Trott et al., 2001) and protein–nucleic acid interactions (Schuck et al., 2013). Numerous proteins with distinct phosphorylation sites have been investigated, and protein kinases affecting a wide range of cellular responses have been characterized (Peck, 2006; Ubersax and Ferrell, 2007; Bond et al., 2011; Dissmeyer and Schnittger, 2011). A growing body of evidence now shows that viral proteins with different functions are phosphorylated by various protein kinases during infection. These include CK2, PKA, PKC, and CK1 protein kinases (Lee and Lucas, 2001; Link et al., 2011; Makarov et al., 2012; Hung et al., 2014), and among these kinases, CK2 phosphorylation effects on infectivity have been most extensively studied.

Protein kinase CK2, a conserved Ser/Thr kinase existing in almost all eukaryotes, phosphorylates proteins with a consensual phosphorylation site motif (S/T-D/E-X-E/D, where X is any residue) (Ubersax and Ferrell, 2007). Increasing evidence indicates that CK2 protein phosphorylation has important roles in plant growth and development (Mulekar et al., 2012), and that CK2 also regulates virus infection processes, including virion assembly, cell-to-cell and long-distance movement, and interactions between viral proteins and other host proteins (Jakubiec and Jupin, 2007; Nardozzi et al., 2010). In addition to the MP phosphorylation effects mentioned in the Introduction, phosphorylation of cucumber mosaic virus, cucumber necrosis virus, and turnip yellow mosaic virus RdRp has substantial effects on virus replication (Kim et al., 2002; Shapka et al., 2005; Stork et al., 2005; Jakubiec et al., 2006; Jakubiec and Jupin, 2007). Ser/Thr phosphorylation has also been suggested to affect the CP functions of several plant viruses. For example, phosphorylation of potato virus A (PVA) CP by host CK2 inhibits viral RNA binding *in vitro*, and mutation of a major phosphorylation CP site generates a PVA variant that is defective in cell-to-cell and long-distance movement (Ivanov et al., 2001, 2003). In addition, phosphorylation of the cauliflower mosaic virus CP precursor at several sites by CK2 is important for virus infectivity and symptom development (Chapdelaine et al., 2002). Phosphorylation of the bamboo mosaic virus CP by CK2 also regulates cell-to-cell movement by modulating RNA binding (Hung et al., 2014).

Although phosphorylation of the PSLV N-terminal portion of the TGB1 protein has been reported (Makarov et al., 2012), but the results were not extended to evaluate the roles of phosphorylation in PSLV movement processes. Our results now demonstrate that the BSMV TGB1 protein is phosphorylated by CK2 *in vitro* and *in planta*, and that the phosphorylation events affect virus movement. Although the prediction programs we used suggest that the $\chi_T$TGB1Thr-395 residue in the $^{399}$DET$^{404}$ site is more likely to be a conserved CK2 phosphorylation site than the Thr-401 $^{401}$DET$^{406}$ site (Meggio and Pinna, 2003), it is noteworthy that Thr-401 localizes within an acidic residue-rich region $^{399}$GET$^{406}$ that may be more favourable for phosphorylation (Battistutta et al., 2000; Riera et al., 2001) than the Thr-395 residue. Thus, based on the *in vitro* phosphorylation assays of the TGB1 mutant derivatives, as well as results derived from LC-MS/MS analysis, we conclude that Thr-401 is the major TGB1 phosphorylation site and that Thr-395 functions as a CK2 docking site and has a more limited phosphorylation role. To the best of our knowledge, this is the first report showing that a plant viral protein, which can be phosphorylated by CK2, has a CK2 docking site adjacent to the phosphorylation site.

To determine the effects of $\chi_T$TGB1 Thr-395 and Thr-401 phosphorylation on virulence, point mutations were introduced into the $\chi_B$BSMV clone. Infectivity results in the monocot and dicot hosts revealed that the mutant derivatives differed in their systemic movement phenotypes. For example, the T395A mutant was the only derivative able to infect *N. benthamiana*, barley, and wheat systemically, but the T395E, T401A, and T401E mutants also systemically infected the monocot hosts (Fig. 5). Our results suggest that the mutants may be compromised by partial disruption of phosphorylation and dephosphorylation dynamics in TGB1 in ways that contribute to diminished cell-to-cell movement. However, the amino acid structures of the T395A, T395D, T395E, T401D, and T401E substitutions are not entirely consistent with this simplistic model, as it is obvious that the substituted amino acids differ in the sizes of their side chains and their charges. Moreover, we cannot exclude the possibility that ‘off-site’ phosphorylation of Thr-395 or phosphorylation by kinases other than CK2 may be elicited by the substitutions and that these events may contribute to protein modifications that affect local and vascular movement.

Hordeivirus TGB1 proteins are multifunctional and contain two positively charged regions rich in lysine (K) and arginine (R) residues at the N-terminal half of the protein and a C-terminal region consisting of seven conserved motifs (I, IA, II, III, IV, V, and VI) (Jackson et al., 2009). The Thr-395 and Thr-401 sites are located between domain IV and V of the TGB1 protein and are not included in the most highly conserved regions. The hordeivirus TGB1 proteins have multiple ssRNA- and dsRNA-binding sites, and hence mutagenesis
of single or closely associated \( \chi_i \) TGB1 protein sites may not have obvious effects on RNA-binding activities \( \textit{in vitro} \).

We have shown previously that the BSMV TGB1 protein is the major protein component of ribonucleoprotein complexes involved in BSMV cell-to-cell movement. TGB1 also participates in interactions of TGB1 and TGB3 proteins during intra- and intercellular virus movement, and functions in TGB1:TGB3 interactions that recruit the TGB2 protein during transport through PD to adjacent cells (Jackson et al., 2009). These interactions are critical for movement because TGB3 serves as a bridge to direct TGB co-localization at the cell wall and to establish close associations with the PD (Lim et al., 2009). Even though both CK2 phosphorylation sites (T395A/T401A) in \( \chi_i \) TGB1 were mutated simultaneously, the mutant TGB1 protein did not elicit obvious changes in subcellular localization patterns (Fig. 7C). However, the \( \chi_i \) TGB1\textsubscript{T395A/T401A} protein did reduce binding affinity to the TGB3 protein compared with the wt TGB1 protein. Therefore, our results provide evidence that CK2 phosphorylation of TGB1 affects BSMV movement by modulating TGB1:TGB3 protein interactions.

In summary, our results shown here provide evidence showing that phosphorylation of the BSMV \( \chi_i \) TGB1 protein by CK2 at C-terminal residues affects cell-to-cell movement and the systemic infection phenotype. Moreover, the mutant results are compatible with a model whereby modulation of TGB1:TGB3 interactions contribute to phenotypic differences with in BSMV movement in monocot and dicot hosts. Compared with the TGB1 proteins of other hordeiviruses (Supplementary Fig. S2A, available at JXB online), Thr-401 but not Thr-395 is conserved in PSLV TGB1 and BSMV TGB1. This implies that the proposed docking site function of Thr-395 may be unique for BSMV phosphorylation. Furthermore, multiple sequence alignments of TGB1 proteins (Supplementary Fig. S2B) showed that the Thr-395 and Thr-401 sites are highly conserved among six sequenced BSMV strains, suggesting that phosphorylation of the TGB1 protein is required during infection of all BSMV strains.

**Supplementary data**

Supplementary data are available at JXB online.

Supplementary Fig. S1. Phosphorylation predictions of the \( \chi_i \) TGB1 protein by the GPS 2.1 program (A) and the ScanSite Motif Scanner online server (B).

Supplementary Fig. S2. Alignment of the TGB1 proteins of the hordeiviruses (A) and among six sequenced BSMV strains (B).

Supplementary Table S1. Primers used in construction and analysis of biologically active BSMV Xinjiang cDNA clones.

Supplementary Table S2. Primers used for site-specific mutagenesis of Xinjiang RNA\( \beta \) clones.

Supplementary Table S3. Sequence alignment of Xinjiang strain RNA\( \alpha \) with different BSMV strains.

Supplementary Table S4. Sequence alignment of Xinjiang strain RNA\( \beta \) with different BSMV strains.

Supplementary Table S5. Sequence alignment of Xinjiang strain RNA\( \gamma \) with different BSMV strains.

Supplementary Table S6. Systemic infectivity efficiency of \( \chi_i \) BSMV TGB1 phosphorylation mutants on dicot and monocot hosts.

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