Mimic Phosphorylation of a β C1 Protein Encoded by TYLCCNB Impairs Its Functions as a Viral Suppressor of RNA Silencing and a Symptom Determinant

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ABSTRACT Phosphorylation of the β C1 protein encoded by the betasatellite of tomato yellow leaf curl China virus (TYLCCNB- β C1) by SNF1-related protein kinase 1 (SnRK1) plays a critical role in defense of host plants against geminivirus infection in *Nicotiana benthamiana*. However, how phosphorylation of TYLCCNB- β C1 impacts its pathogenic functions during viral infection remains elusive. In this study, we identified two additional tyrosine residues in TYLCCNB- β C1 that are phosphorylated by SnRK1. The effects of TYLCCNB- β C1 phosphorylation on its functions as a viral suppressor of RNA silencing (VSR) and a symptom determinant were investigated via phosphorylation mimic mutants in *N. benthamiana* plants. Mutations that mimic phosphorylation of TYLCCNB- β C1 at tyrosine 5 and tyrosine 110 attenuated disease symptoms during viral infection. The phosphorylation mimics weakened the ability of TYLCCNB- β C1 to reverse transcriptional gene silencing and to suppress posttranscriptional gene silencing and abolished its interaction with *N. benthamiana* ASYMMETRIC LEAVES 1 in *N. benthamiana* leaves. The mimic phosphorylation of TYLCCNB- β C1 had no impact on its protein stability, subcellular localization, or self-association. Our data establish an inhibitory effect of phosphorylation of TYLCCNB- β C1 on its pathogenic functions as a VSR and a symptom determinant and provide a mechanistic explanation of how SnRK1 functions as a host defense factor.

IMPORTANCE Tomato yellow leaf curl China virus (TYLCCNV), which causes a severe yellow leaf curl disease in China, is a monopartite geminivirus associated with the betasatellite (TYLCCNB). TYLCCNB encodes a single pathogenicity protein, β C1 (TYLCCNB- β C1), which functions as both a viral suppressor of RNA silencing (VSR) and a symptom determinant. Here, we show that mimicking phosphorylation of TYLCCNB- β C1 weakens its ability to reverse transcriptional gene silencing, to suppress posttranscriptional gene silencing, and to interact with *N. benthamiana* ASYMMETRIC LEAVES 1. To our knowledge, this is the first report establishing an inhibitory effect of phosphorylation of TYLCCNB- β C1 on its pathogenic functions as both a VSR and a symptom determinant and to provide a mechanistic explanation of how SNF1-related protein kinase 1 acts as a host defense factor. These findings expand the scope of phosphorylation-mediated defense mechanisms and contribute to further understanding of plant defense mechanisms against geminiviruses.

KEYWORDS TYLCCNB- β C1, *Nicotiana benthamiana*, SnRK1, geminivirus, host defense factor, posttranscriptional gene silencing, protein phosphorylation, transcriptional gene silencing

Received 24 February 2017 Accepted 17 May 2017

Accepted manuscript posted online 24 May 2017

Citation Zhong X, Wang ZQ, Xiao R, Cao L, Wang Y, Xie Y, Zhou X. 2017. Mimic phosphorylation of a β C1 protein encoded by TYLCCNB impairs its functions as a viral suppressor of RNA silencing and a symptom determinant. *J Virol* 91:e00300-17. <https://doi.org/10.1128/JVI.00300-17>.

Editor Anne E. Simon, University of Maryland, College Park

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The *Geminiviridae* are a diverse family of plant viruses that are able to infect food and cash crops, causing serious crop failure and economic losses worldwide (1–5). The family *Geminiviridae* is currently divided into nine genera, *Becurtovirus*, *Begomovirus*, *Curtovirus*, *Eragrovirus*, *Mastrevirus*, *Topocuvirus*, *Turncurtovirus*, *Capulavirus*, and *Grablovirus*, based on genome organization, host range, and insect vector (6). Whitefly-transmitted begomoviruses, with more than 200 species, constitute the largest genus of geminiviruses (4, 7). The genomes of begomoviruses can be either monopartite or bipartite (with genomic components referred to as DNA-A and DNA-B), with a length of approximately 2.7 kb (1, 8, 9). Over evolutionary time, the monopartite begomoviruses have acquired two classes of satellite molecules, known as betasatellite (DNA β) and alphasatellite (DNA1), which act to decrease host resistance, move virus particles, and enhance infection (5, 10). The first DNA satellite was identified with tomato leaf curl virus (ToLCV) (11); since then, many monopartite begomovirus species have been frequently found to be associated with betasatellites, and the number of begomoviruses associated with betasatellites is continuously increasing (5).

Tomato yellow leaf curl China virus (TYLCCNV), which causes a severe yellow leaf curl disease in tomato and tobacco in China, is a monopartite begomovirus in association with a betasatellite (TYLCCNB) (12, 13). The genome of TYLCCNB is approximately half the size of that of its helper virus TYLCCNV and is required for induction of leaf curl disease in plants (13). It encodes a single pathogenicity protein, TYLCCNB- β C1, which functions as a viral suppressor of RNA silencing (VSR) and a symptom determinant (13–15). Our previous studies have shown that in both *Nicotiana* (host) and *Arabidopsis* (nonhost) plants, overexpression of TYLCCNB- β C1 produces virus-like symptoms, including leaf curling, vein swelling, and blistering of leaves (13–15). TYLCCNB- β C1 also upregulates an endogenous RNA silencing suppressor *Nicotiana benthamiana calmodulin-like protein* gene (*Nbrgs-CaM*), and *Nbrgs-CaM* regulates RNA silencing and promotes geminivirus infection by repressing the expression of *RNA-dependent RNA polymerase 6* (*RDR6*) and promoting *N. benthamiana* suppressor of gene silencing 3 (*NbSGS3*) degradation via the autophagy pathway (16, 17). TYLCCNB- β C1 is also involved in suppressing transcriptional gene silencing (TGS) (18), which serves as a robust defense strategy against geminiviruses (19). Furthermore, TYLCCNB- β C1 interacts with ASYM-METRIC LEAVES 1 (AS1) to alter leaf development in *Arabidopsis* (14). TYLCCNB- β C1 can also repress the jasmonic acid (JA)-mediated plant defenses against the whitefly, thereby accelerating its population increase (20). Therefore, it is necessary to clarify the molecular mechanisms underlying the pathogenesis of TYLCCNB- β C1.

Studies during the past decade have led to significant advances in our understanding of plant defenses against geminivirus infections. Host plants have developed at least four different defense strategies to deal with geminivirus infection. The first line of defense is posttranscriptional gene silencing (PTGS)-mediated RNA interference (RNAi) that can aggressively decrease geminivirus transcripts (4, 16, 17, 19, 21). RNA-dependent DNA methylation (RdDM)-mediated TGS also functions as a defense mechanism against geminivirus chromatin (5, 22–24). Recently, *R* gene-mediated resistance has been proposed as another essential defense strategy against geminiviruses. For example, the begomovirus nuclear shuttle protein (NSP)-interacting kinase 1 (NIK1) is able to activate antiviral immunity against cabbage leaf curl virus (CaLCuV) (25, 26). Besides the above-mentioned defense mechanisms, host factors such as SNF1-related protein kinase 1 (SnRK1) have also been involved in defense against geminiviruses (27–29).

Protein kinases phosphorylate serine (Ser), threonine (Thr), or tyrosine (Tyr) residues of target proteins to alter their enzymatic activity, cellular localization, and interaction with other proteins, as well as other biochemical properties (28). Protein phosphorylation plays crucial roles in signal transduction in developmental and environmental responses and is vital in modulating plant-pathogen interactions. Plant SnRK1, which is homologous to AMPK and SNF1 in animals and *Saccharomyces cerevisiae*, respectively, acts as a central integrator of metabolic homeostasis in plants and a critical regulator of diverse stress responses triggered by viral, bacterial, and fungal infections or even

herbivores (29, 30). Recently we and others have shown that TYLCCNB- β C1 and begomovirus AL2/C2 proteins are targets for plant SnRK1, which plays critical roles in defense against geminivirus infections (27, 28). Overexpression of SnRK1 in *N. benthamiana* plants attenuates virus symptoms and decreases viral DNA accumulation, whereas knockdown of SnRK1 results in increased susceptibility to infection (27, 31). SnRK1 is upregulated by TYLCCNB and phosphorylates TYLCCNB- β C1 at Ser-33 and Thr-78 (27).

Here, we show that two Tyr residues in TYLCCNB- β C1 are also phosphorylation targets for SnRK1 and that mutations in four residues of TYLCCNB- β C1 that mimic phosphorylation impair its pathogenic functions as a VSR and a symptom determinant. Phosphorylation of TYLCCNB- β C1 weakened its ability to reverse TGS and suppress PTGS and abolished its interaction with *N. benthamiana* AS1 (NbAS1). Our findings give insights into the molecular mechanisms of host phosphorylation of TYLCCNB- β C1, which appears to be a critical defense strategy against geminivirus infection, and may provide practical strategies for viral disease management.

RESULTS

Identification of two novel Tyr phosphorylation sites of TYLCCNB- β C1. Our previous study showed that Ser-33 and Thr-78 in TYLCCNB- β C1 are two important functional phosphorylation sites phosphorylated by SnRK1. A double-phosphorylation mimic aspartate (D) mutant, TYLCCNB- β C1-S33D/T78D (β C1-2D), shows delayed and attenuated disease symptoms and lower levels of viral DNA accumulation in systemically infected leaves when infected with TYLCCNV isolate Y10 and its associated TYLCCNB (Y10A/ β) (27). However, TYLCCNB- β C1 protein with alanine (A) substitutions for Ser-33 and Thr-78 (β C1-S33A/T78A [β C1-2A]) retained low (37% to 28% of the wild-type [WT] TYLCCNB- β C1 protein [β C1-WT]) but measurable phosphorylation signals (27), suggesting that other potential SnRK1 phosphorylation sites exist in TYLCCNB- β C1.

To test whether additional residues within TYLCCNB- β C1 are phosphorylated by SnRK1, we analyzed the primary amino acid sequence of TYLCCNB- β C1 using the NetPhos 2.0 server (<http://www.cbs.dtu.dk/services/NetPhos>). The analysis revealed that Tyr-5 and Tyr-110 are putative phosphorylation sites, in addition to Ser-33 and Thr-78 (Fig. 1A). To experimentally confirm the bioinformatic prediction, we mutated Tyr-5 and Tyr-110 to phenylalanine (F), alone or in conjunction with β C1-2A, to generate triple mutants (β C1-2A-Y5F and β C1-2A-Y110F) or a quadruple mutant (β C1-2A-Y5F/Y110F [β C1-2A/2F]) (Fig. 1B). Remarkably, β C1-2A/2F exhibited little if any autoradiographic signal, and β C1-2A-Y5F and β C1-2A-Y110F exhibited reduced signals compared with β C1-WT (Fig. 1C and D). Furthermore, we also mutated Tyr-5 and Tyr-110 to glutamate (E), alone or in conjunction with β C1-2D, to generate triple mutants (β C1-2D-Y5E and β C1-2D-Y110E) or a quadruple mutant (β C1-2D-Y5E/Y110E [β C1-2D/2E]) and examined Tyr-5 and Tyr-110 in TYLCCNB- β C1 in *N. benthamiana* plants by immunoprecipitation, followed by antibody against phosphorylated tyrosine as described previously (32). As shown in Fig. 1E, compared with β C1-WT, β C1-2D-Y5E and β C1-2D-Y110E exhibited reduced immunologic signals and β C1-2D/2E exhibited little if any signal. These results suggest that Tyr-5 and Tyr-110 are actual Tyr phosphorylation sites in the TYLCCNB- β C1 protein that are phosphorylated by SnRK1.

Mutation of Tyr-5 and Tyr-110 of TYLCCNB- β C1 to mimic phosphorylation attenuates virus symptoms. As our previous study had shown that mutants of TYLCCNB- β C1 that mimic phosphorylation of Ser-33 and Thr-78 can slow down development of Y10A/ β -induced disease symptoms (27), we tested whether Tyr-5 and Tyr-110 of TYLCCNB- β C1 are also involved in symptom development during Y10A/ β infection. To do this, we mutated Tyr-5 and Tyr-110 to glutamate (E), either alone or together, to generate phosphorylation mimic infectious clones (β -Y5E, β -Y110E, and β -2E) (Fig. 2A). Wild-type *N. benthamiana* plants were agroinfiltrated with wild-type TYLCCNB (β -WT) or the phosphorylation mimics in combination with their helper virus, TYLCCNV (Y10A). As shown in Fig. 2B, iii, plants inoculated with Y10A/ β -WT developed

FIG 1 Tyr-5 and Tyr-110 phosphorylation sites of TYLCCNB-βC1. (A) Prediction of potential phosphorylation sites in the TYLCCNB-βC1 protein sequence using NetPhos 2.0. (B) Schematic presentation of βC1-WT and mutant TYLCCNB-βC1 proteins. (C) Tyr-5 and Tyr-110 of TYLCCNB-βC1 are phosphorylated by SnRK1 *in vitro*. The *in vitro* kinase assay was performed using GST-SnRK1-KD (kinase domain) as a kinase, GST-GRIK as a kinase to active SnRK1-KD, and wild-type or mutant TYLCCNB-βC1 proteins as substrates. Phosphorylation was analyzed by autoradiography (top), and the protein loading was shown by Coomassie brilliant blue (CBB) staining (bottom). (D) The radioactive signals shown in panel C were quantified with Quantity One Software (Bio-Rad). The data are shown as means and SD of three biological replicates. Means with different letters are significantly different (Tukey's test; $P < 0.05$). (E) Tyr-5 and Tyr-110 of TYLCCNB-βC1 are phosphorylated by SnRK1 *in vivo*. Phosphorylation of Tyr-5 and Tyr-110 on wild-type or mutant TYLCCNB-βC1-GFP proteins immunoprecipitated from *N. benthamiana* leaves. IP was performed with an antibody to GFP (IP: α-GFP), and Tyr-5 and Tyr-110 were analyzed by WB with an antibody against phosphorylated tyrosine (WB: α-pTyr). Inputs of GFP and wild-type or mutant TYLCCNB-βC1-GFP proteins are shown by WB (middle gel). WB analysis was carried out using an antibody to actin for a loading control (bottom gel). The experiment was repeated three times with similar results.

disease symptoms characterized by severe curling of leaves and twisted shoots at 21 days postinoculation (dpi). In contrast, plants inoculated with Y10A/β-Y5E, Y10A/β-Y110E, or Y10A/β-2E displayed moderate leaf curling without shoot twisting (Fig. 2B, iv to vi). It is interesting that *N. benthamiana* plants inoculated with Y10A/β-Y110E showed upward curling, which is opposite to the downward curling induced by

FIG 2 Effects of mutants in phosphorylation sites of TYLCCNB- β C1 on virus infection. (A) Schematic representation of the infectious clones of β -WT and the phosphorylation mimic mutants, β -Y5E, β -Y110E, β -2E, β -2D, β -2D-Y5E, β -2D-Y110E, and β -2D/2E, used in panel B. (B) Symptoms observed in wild-type *N. benthamiana* plants agroinoculated with infectious clones of TYLCCNBV (Y10A), alone or associated with WT or mutant TYLCCNB, at 21 dpi. Mock indicates plants agroinoculated with an empty-vector control (pBINPLUS). (C) Time course of infection in wild-type *N. benthamiana* plants inoculated with Y10A in association with WT or mutant infectious clones. The values represent the percentages of systemically infected plants at different dpi. The data are given as means $\pm$ SD of three biological replicates. (D and E) Relative accumulation levels of Y10A (D) and TYLCCNB or its phosphorylation mimic mutants (E) in agroinoculated plants. Viral accumulation was determined by qPCR at 21 dpi, as described for panel B. The values represent viral DNA accumulation relative to levels in control groups (*N. benthamiana* plants infected with Y10A/ β -WT), the values of which are set to 100%. The data are shown as means $\pm$ SD of three biological replicates. Means with different letters are significantly different (Tukey's test; $P < 0.05$). ND, not detectable.

Y10A/ β -WT (Fig. 2B, v). These results suggest that Tyr-5 and Tyr-110 are associated with the pathogenicity of TYLCCNB- β C1.

To investigate the additive effects of S33D/T78D and Y5E/Y110E on TYLCCNB- β C1 pathogenicity, we further mutated Tyr-5 and Tyr-110 to glutamate, alone or together with the double mutation of β C1-S33D/T78D (β -2D), to generate infectious clones containing triple (β -2D-Y5E and β -2D-Y110E) or quadruple (β -2D-Y5E/Y110E [β -2D/2E]) phosphorylation mimic mutants (Fig. 2A). At 21 dpi, plants agroinfiltrated with Y10A/ β -2D-Y5E or Y10A/ β -2D-Y110E showed only slight leaf curling without shoot twisting (Fig. 2B, viii and ix). Moreover, *N. benthamiana* plants inoculated with Y10A/ β -2D/2E did not display any disease symptoms up to 21 dpi (Fig. 2B, x). These results suggest that phosphorylation of Tyr-5 and Tyr-110 has an additive effect with phosphorylation of Ser-33 and Thr-78 to weaken TYLCCNB- β C1 pathogenicity.

We also investigated the infection course of Y10A associated with β -WT or its phosphorylation mimic mutants in wild-type *N. benthamiana* plants, as described

FIG 3 Effects of phosphorylation mimic mutants of TYLCCNB-βC1 on symptom modulation and protein stability. (A) Schematic representation of PVX expression constructs of wild-type and phosphorylation mimic mutants (PVX:βC1-WT, PVX:βC1-2D, PVX:βC1-2D-Y5E, PVX:βC1-2D-Y110E, and PVX:βC1-2D/2E) of TYLCCNB-βC1 used in panel B. Empty PVX vector (PVX:Vec) was used as the negative control. (B) Symptoms of wild-type *N. benthamiana* plants agroinfiltrated with different PVX expression constructs at 10 dpi. Mock indicates plants agroinfiltrated with the control construct PVX:Vec. (C) WB analysis of wild-type and phosphorylation mimic mutants of TYLCCNB-βC1 in *N. benthamiana* plants shown in panel B. Total soluble proteins were extracted from systemically infected leaves. The PVX coat protein (CP) was used as a loading control. The experiments were repeated three times with similar results.

previously (27, 33). For Y10A/β-WT inoculation, disease symptoms started to appear at 3 or 4 dpi, with all the plants displaying typical symptoms at 8 to 10 dpi (Fig. 2C). In contrast, symptom appearance was delayed for plants coinoculated with infectious clones containing phosphorylation mimic Y10A/β-2D, Y10A/β-2D-Y5E, or Y10A/β-2D-Y110E, which was especially obvious in the quadruple mutant Y10A/β-2D/2E (Fig. 2C). We further compared viral DNA accumulation in systemically infected leaves of plants infected with Y10A associated with β-WT or its phosphorylation mimic mutants using quantitative PCR (qPCR). As shown in Fig. 2D, there was no significant difference in the accumulation of helper virus DNA (Y10A) in plants infected with the Y10A/β-WT or Y10A/β phosphorylation mimic mutants, suggesting that phosphorylation of TYLCCNB-βC1 has little or no impact on the accumulation of its helper virus. However, accumulation of TYLCCNB (β) was lower in plants coinoculated with infectious clones containing phosphorylation mimic Y10A/β-2D-Y5E, Y10A/β-2D-Y110E, or Y10A/β-2D/2E than that detected in plants infected with Y10A/β-WT (Fig. 2E). Together, these results suggest that Tyr-5 and Tyr-110 of TYLCCNB-βC1 are functional phosphorylation sites, and mutations that mimic phosphorylation at Tyr-5 and Tyr-110 attenuate virus infection symptoms.

To further validate our results for the phosphorylation mimics of TYLCCNB-βC1 from the infectious clones and to determine whether the reduced virus symptoms were related to the stability of the TYLCCNB-βC1 protein, the open reading frames (ORFs) of βC1-WT or its phosphorylation mimic mutants were cloned into a potato virus X (PVX)-based vector (34) (Fig. 3A). As shown in Fig. 3B, the phosphorylation mimic mutations of TYLCCNB-βC1 weakened symptoms in *N. benthamiana* plants inoculated with the PVX-based constructs. The triple (PVX:βC1-2D-Y5E or PVX:βC1-2D-Y110E) and quadruple (PVX:βC1-2D/2E) phosphorylation mimic mutants greatly abated the leaf curl and petiole elongation associated with wild-type PVX:βC1-WT (Fig. 3B). These observations are consistent with the results obtained using infectious clones. Furthermore, we measured the accumulations of wild-type and mutant TYLCCNB-βC1 proteins

in wild-type *N. benthamiana* plants, as shown in Fig. 3B, by Western blotting (WB). As shown in Fig. 3C, accumulations of the wild-type and mutant TYLCCNB- β C1 were very similar in *N. benthamiana* plants. Collectively, these data suggest that mimic phosphorylation of TYLCCNB- β C1 has no apparent impact on its protein stability and that the weakened virus symptoms observed are not a consequence of reduced stability of TYLCCNB- β C1 protein.

Subcellular localization and self-interaction of phosphorylation mimic mutants of TYLCCNB- β C1. To gain insight into how phosphorylation of TYLCCNB- β C1 attenuates disease symptoms during virus infection, we determined the subcellular localization of phosphorylation mimic mutants of TYLCCNB- β C1 (β C1-2D and β C1-2D/2E). The ORFs of β C1-2D and β C1-2D/2E were cloned by translational fusions with green fluorescent protein (GFP) at the N or C terminus of GFP and expression of the chimeric proteins under the control of a CaMV 35S (35S) promoter (35S: β C1-2D-GFP, 35S:GFP- β C1-2D, 35S: β C1-2D/2E-GFP, and 35S:GFP- β C1-2D/2E). *N. benthamiana* leaves agroinfiltrated with 35S:GFP, 35S: β C1-WT-GFP, or 35S:GFP- β C1-WT were used as controls. *N. benthamiana* epidermal cells transiently expressing GFP only (35S:GFP) showed fluorescence throughout the cells (Fig. 4A). In agreement with our previous observations, diffuse fluorescence was detected in both the cytosol and nucleus in leaf cells expressing β C1-WT-GFP and GFP- β C1-WT (Fig. 4B and E), indicating that β C1-WT is localized to both compartments (35). Similarly, fluorescence was also detected in the cytosol and nucleus in leaf cells expressing β C1-2D-GFP, GFP- β C1-2D, β C1-2D/2E-GFP, and GFP- β C1-2D/2E (Fig. 4C and F, and D and G). These results, therefore, suggest that mutants that mimic phosphorylation of TYLCCNB- β C1 have no effect on subcellular localization of TYLCCNB- β C1.

It has been shown that oligomerization of TYLCCNB- β C1 is critical to its function as a pathogenicity determinant (35). We next determined whether phosphorylation mimics of TYLCCNB- β C1 affect its oligomerization, using a bimolecular fluorescence complementation (BiFC) assay as described previously (36). *N. benthamiana* leaves were coagroinfiltrated with constructs designed to express β C1-WT, β C1-2D, and β C1-2D/2E fused at their N or C termini with the N- or C-terminal portions of a yellow fluorescent protein (YFP) (2YN and 2YC). Leaves were photographed 48 h after coinfiltration using a confocal laser scanning microscope. *N. benthamiana* leaves coagroinfiltrated with the empty vectors 2YN and 2YC were used as a negative control (Fig. 5A). Consistent with our previous observation, YFP fluorescence was detected in the cytosol and nucleus in *N. benthamiana* epidermal cells expressing 2YN: β C1-WT and 2YC: β C1-WT (Fig. 5B), indicating oligomerization of β C1-WT. Similarly, YFP fluorescence was detected in the cytosol and nucleus in *N. benthamiana* epidermal cells expressing 2YN: β C1-2D and 2YC: β C1-2D or 2YN: β C1-2D/2E and 2YC: β C1-2D/2E (Fig. 5C and D). This suggests that β C1-2D and β C1-2D/2E are still able to form multimeric complexes even when mimic phosphorylated. Together, these data suggest that mutations that mimic phosphorylation of TYLCCNB- β C1 do not affect the ability of TYLCCNB- β C1 to oligomerize.

Mimic phosphorylation of TYLCCNB- β C1 affects its ability to reverse established methylation-mediated TGS. Our previous study showed that TYLCCNB- β C1 suppresses methylation-mediated TGS in *N. benthamiana* plants during Y10A/ β infection (18). We next asked whether phosphorylation mimic mutants of TYLCCNB- β C1 affect its ability to reverse established methylation-mediated TGS. To determine this, we used transgenic *N. benthamiana* 16-TGS plants, which contain a GFP transgene that is downstream of a transcriptionally silenced 35S promoter (37, 38). 16-TGS seedlings were inoculated with Y10A alone or in conjunction with β -WT or the phosphorylation mimic mutants (β -2D and β -2D/2E). Consistently, the 16-TGS seedlings infected with Y10A alone or with β -2D and β -2D/2E showed very slight disease symptoms, while severe disease symptoms were observed in seedlings infected with Y10A/ β -WT at 14 dpi (Fig. 6A). Under UV light, GFP fluorescence was obviously visible in veins and petioles of symptomatic leaves of 16-TGS plants infected with Y10A/ β -WT at 14 dpi. However, 16-TGS seedlings infected with Y10A alone or with β -2D and β -2D/2E did not display any GFP fluorescence (Fig. 6A). As expected, qPCR and WB analyses showed that

FIG 4 Subcellular localization of β C1-WT and phosphorylation mimic mutants (β C1-2D and β C1-2D/2E) of TYLCCNB- β C1. (A) Localization of GFP fluorescence in epidermal cells of *N. benthamiana*. (B and E) Localization of GFP fluorescence from β C1-WT protein fused to the N- and C termini of GFP, respectively. (C and F) Localization of GFP fluorescence from β C1-2D protein fused to the N- and C termini of GFP, respectively. (D and G) Localization of GFP fluorescence from β C1-2D/2E protein fused to the N- and C termini of GFP, respectively. The cells were photographed 48 h after infiltration using a confocal laser scanning microscope. RFP-histone 2B (RFP) was used as a marker for the nucleus. GFP, GFP fluorescence; RFP, RFP fluorescence; Bright, bright-field images; Merged, merged images. Bars, 50 μ m.

the lack of visible fluorescence in 16-TGS plants infected with Y10A/ β -2D or Y10A/ β -2D/2E was due to a deficiency in the accumulation of GFP mRNA and protein (Fig. 6B and C). These results suggest that the phosphorylation mimic mutants of TYLCCNB- β C1 are unable to reverse established methylation-mediated TGS in *N. benthamiana* plants.

To further confirm the effect of phosphorylation mimics of TYLCCNB- β C1 on demethylation, a routine chop-PCR (39) was employed to determine the methylation status of the 35S promoter in 16-TGS plants infected with Y10A alone or with β -WT or its phosphorylation mimic mutants (β -2D and β -2D/2E). Total nucleic acids were extracted from vascular tissue and digested with a methylation-sensitive endonuclease, HinfI, or a methylation-dependent endonuclease, McrBC, and then subjected to PCR. Levels of PCR product derived from the 35S promoter in 16-TGS seedlings infected with Y10A/ β -WT were much lower than those detected in plants infected by Y10A/ β -2D or

FIG 5 Self-interaction of β C1-WT and phosphorylation mimic mutants (β C1-2D and β C1-2D/2E) of TYLCCNB- β C1 in epidermal cells of *N. benthamiana* by BiFC. *N. benthamiana* leaves were coagroinfiltrated with 2YN and 2YC empty vectors (A), 2YN: β C1-WT and 2YC: β C1-WT (B), 2YN: β C1-2D and 2YC: β C1-2D (C), or 2YN: β C1-2D/2E and 2YC: β C1-2D/2E (D). The cells were photographed 48 h after coinfiltration using a confocal laser scanning microscope. RFP-histone 2B (RFP) was used as a marker for the nucleus. YFP, YFP fluorescence; RFP, RFP fluorescence; Bright, bright-field images; Merged, merged images. Bars, 50 μ m.

Y10A/ β -2D/2E when using the endonuclease *Hinf*I. In contrast, restriction with the endonuclease *Mcr*BC produced the opposite outcome (Fig. 6D). These data indicate that the 35S promoter is methylated during infection of 16-TGS plants with Y10A/ β -2D or Y10A/ β -2D/2E. Taken together, these results suggest that phosphorylation mimic mutants of TYLCCNB- β C1 are not able to reverse established methylation-mediated TGS in *N. benthamiana* plants.

Mimic phosphorylation of TYLCCNB- β C1 weakens its suppression of PTGS. Our previous studies have also shown that TYLCCNB- β C1 is involved in suppressing PTGS in *N. benthamiana* plants during Y10A/ β infection, thus counteracting RNAi-based antiviral responses (15–17). We further examined whether phosphorylation mimics of TYLCCNB- β C1 affect its ability to suppress PTGS. For this purpose, we used a transient silencing suppression assay based on GFP transgenic *N. benthamiana* 16c plants (16, 40). In this assay, *Agrobacterium* containing a binary vector designed to transiently express sense GFP (35S:GFP) and *Agrobacterium* harboring a candidate suppressor gene were coinfiltrated into leaves of 16c plants. *Agrobacterium* containing an empty pCHF3 vector (pCHF3:Vec) and *Agrobacterium* containing tomato bushy stunt virus (TBSV) P19 ORF (P19) (41, 42) were used as negative and positive controls, respectively. Consistently, leaves of 16c seedlings coinfiltrated with *Agrobacterium* containing 35S:GFP and β C1-WT (pCHF3: β C1-WT) elicited relatively strong green GFP fluorescence as a consequence of suppression of GFP RNA silencing (Fig. 7A). However, leaves coinfiltrated with *Agrobacterium* harboring a phosphorylation mimic mutant (pCHF3: β C1-2D or pCHF3: β C1-2D/2E) and 35S:GFP displayed very faint GFP fluorescence, similar to the negative control (Fig. 7A), indicating that the GFP RNA was degraded. As anticipated, qPCR and WB analyses verified that lower fluorescence observed in leaves of 16c plants coinfiltrated with the phosphorylation mimic mutants (pCHF3: β C1-2D and pCHF3: β C1-2D/2E), together with 35S:GFP, was due to reduced accumulation of GFP mRNA and protein (Fig. 7B and C). Collectively, these results suggest that mimic phosphorylation of TYLCCNB- β C1 weakens its suppression of PTGS in *N. benthamiana* plants.

FIG 6 Mimicking phosphorylation of TYLCCNB-βC1 impacts its ability to reverse TGS of a *GFP* transgene and suppress cytosine methylation. (A) Transgenic *N. benthamiana* 16-TGS plants were agroinfiltrated with TYLCCNV (Y10A) alone or in conjunction with β-WT or the phosphorylation mimic mutants (β-2D and β-2D/2E), and the plants were photographed under white light or UV light at 14 dpi. 16-TGS plants agroinoculated with Y10A alone were used as negative controls. (B) qPCR analysis of *GFP* mRNA accumulation in systemically infected leaves shown in panel A. The level of gene expression was normalized to that of *NbACT2*; the values represent relative *GFP* mRNA accumulation compared with mRNA from 16-TGS plants infected with Y10A/β-WT (100%). The data are shown as means and SD of three biological replicates. Means with different letters are significantly different (Tukey's test; $P < 0.05$). (C) Western blot assay of *GFP* protein accumulation in systemically infected leaves shown in panel A. Coomassie brilliant blue (CBB) staining of the large subunit of Rubisco was used as a loading control. (D) Analysis of DNA methylation at the 35S promoter using chop-PCR. Genomic DNA was extracted from the systemically infected leaves shown in panel A. Samples from 16-TGS plants agroinoculated with Y10A alone were used as the negative control. The experiments were repeated three times with similar results.

Phosphorylation mimics of TYLCCNB-βC1 weaken PTGS suppression at the level of *Nbrgs-CaM*. We next investigated how phosphorylation mimics of TYLCCNB-βC1 weaken PTGS suppression in *N. benthamiana* plants. Our previous study demonstrated that TYLCCNB-βC1 is able to upregulate *Nbrgs-CaM* to repress expression of *RDR6* and to suppress PTGS (16). We therefore determined whether phosphorylation mimics of TYLCCNB-βC1 affect the upregulation of *Nbrgs-CaM* using qPCR. As shown in Fig. 8A, *Nbrgs-CaM* was upregulated ~6-fold in response to βC1-WT. The phosphorylation mimic mutants (βC1-2D and βC1-2D/2E) were deficient in their ability to upregulate *Nbrgs-CaM*, with βC1-2D exhibiting an ~2-fold reduction and βC1-2D/2E a

FIG 7 Mimicking phosphorylation of TYLCCNB- β C1 impairs its ability to suppress PTGS. (A) Suppression of PTGS of GFP in leaves of transgenic *N. benthamiana* 16c plants. Leaves of 16c plants were coagroinfiltrated with constructs harboring GFP (35S:GFP) and either a pCHF3 vector control (pCHF3:Vec), TBSV P19 (P19), wild-type TYLCCNB- β C1 (pCHF3:βC1-WT), or phosphorylation mimic mutants (pCHF3:βC1-2D and pCHF3:βC1-2D/2E). The agroinfiltrated leaves were photographed under UV light at 6 dpi. (B) qPCR analysis of GFP mRNA accumulation in agroinfiltrated leaf patches shown in panel A. The level of gene expression was normalized to that of *NbACT2*; the values represent relative GFP mRNA accumulation compared to mRNA from 16c plants infected with P19 (100%). The data are shown as means and SD of three biological replicates. Means with different letters are significantly different (Tukey's test; $P < 0.05$). (C) Western blot assay of GFP accumulation in agroinfiltrated leaf patches shown in panel A. CBB staining of the large subunit of Rubisco was used as a loading control. The experiment was repeated three times with similar results.

complete loss of upregulation. These data suggest that mutants that mimic phosphorylation of TYLCCNB- β C1 reduce its ability to upregulate *Nbrgs-CaM*. In parallel, transient transcriptional activation of the *Nbrgs-CaM* promoter by β C1-WT or its phosphorylation mimic mutants (β C1-2D and β C1-2D/2E) was examined using a dual-luciferase system in agroinfiltrated *N. benthamiana* leaves. The ORFs of β C1-WT and its phosphorylation mimic mutants (β C1-2D and β C1-2D/2E) were cloned into the binary vector pCHF3 to serve as effectors. A 1.5-kb (relative to the ATG at bp +1) fragment containing the *Nbrgs-CaM* promoter was cloned into pGreenII0800-LUC upstream of firefly luciferase (LUC) to serve as a reporter, with 35S promoter-driven *Renilla* luciferase (REN) as the internal control (Fig. 8B). Consistently, coexpression of β C1-WT with the LUC reporter significantly increased the LUC/REN ratio (Fig. 8C), indicating that β C1-WT functions as a transcription activator of *Nbrgs-CaM*. However, compared with β C1-WT, coexpression of β C1-2D or β C1-2D/2E with the LUC reporter showed reduced LUC/REN ratios (Fig. 8C), indicating that they had partially or totally lost the capacity to upregulate *Nbrgs-CaM*. Taken together, these results suggest that mimic phosphorylation of TYLCCNB- β C1 weakens its suppression of PTGS, most likely at the level of *Nbrgs-CaM*.

Interaction of TYLCCNB- β C1 with NbAS1 is abolished in phosphorylation mimic mutants. It has been shown that TYLCCNB- β C1 interacts with AS1 to alter leaf development and phenocopies virus-induced disease symptoms in transgenic *Arabidopsis* expressing the pathogenicity determinant TYLCCNB- β C1 (14). In our earlier experiments, we demonstrated that phosphorylation mimic mutants of TYLCCNB- β C1 attenuate virus infection symptoms in *N. benthamiana* plants (Fig. 2). We asked whether β C1-WT also interacts with AS1 in tobacco plants to induce disease symptoms and if this interaction is impaired for its phosphorylation mimic mutants (β C1-2D and β C1-

FIG 8 Diminished suppression of PTGS by phosphorylation mimic mutants of TYLCCNB-βC1 occurs at the level of *Nbrgs-CaM*. (A) *Nbrgs-CaM* mRNA levels in wild-type *N. benthamiana* plants agroinfiltrated with PVX:βC1-WT, PVX:βC1-2D, or PVX:βC1-2D/2E at 7 dpi. An empty PVX vector (PVX:Vec) was used as the negative control. The level of gene expression was normalized to that of *NbGAPDH*, and the values represent relative *Nbrgs-CaM* mRNA accumulation levels compared to the mRNA level in *N. benthamiana* plants infected with PVX:Vec (1.0). The data are shown as means $\pm$ SD of three biological replicates. Means with different letters are significantly different (Tukey's test; $P < 0.05$). (B) Schematic diagram showing the constructs used in the transient transcriptional activity assay in panel C. The ORFs of βC1-WT and phosphorylation mimic mutants (βC1-2D and βC1-2D/2E) were cloned into the binary vector, pCHF3, to serve as effectors. Empty pCHF3 vector (pCHF3:Vec) was used as the negative control. A 1.5-kb (relative to the ATG at bp +1) promoter of *Nbrgs-CaM* was used in the transient transcriptional activity assay. (C) The *Nbrgs-CaM* promoter can be activated by βC1-WT, but not by the phosphorylation mimic mutant βC1-2D/2E. The P_{NbCaM} :LUC reporter was coagroinfiltrated with the indicated effector constructs. The LUC/REN ratio represents the P_{NbCaM} :LUC activity relative to the internal control (REN driven by the 35S promoter). The data are shown as means $\pm$ SD of three biological replicates. Means with different letters are significantly different (Tukey's test; $P < 0.05$).

FIG 9 Mimicking phosphorylation of TYLCCNB- β C1 weakens its interaction with NbAS1. (A) *In vivo* interactions of β C1-WT and phosphorylation mimic mutants (β C1-2D and β C1-2D/2E) with NbAS1 in *N. benthamiana*. (Top) *N. benthamiana* leaves were coagroinfiltrated with the indicated expression constructs, CoIP was performed with an antibody to Flag (IP: α -Flag), and the proteins were analyzed by WB with an antibody to GFP (WB: α -GFP). β C1-WT, β C1-2D, and β C1-2D/2E coimmunoprecipitated with NbAS1. (Middle-two gels) Inputs of NbAS1-Flag and β C1-WT-GFP, β C1-2D-GFP, β C1-2D/2E-GFP, or GFP are shown by WB. (Bottom) WB analysis was carried out using an antibody to actin for a loading control. (B) Interaction of TYLCCNB- β C1 and NbAS1 is largely abolished by SnRK1. (Top) *N. benthamiana* leaves were coagroinfiltrated with the indicated expression constructs in conjunction with different concentrations (OD₆₀₀) of SnRK1, as indicated. CoIP was performed with an antibody to Flag (IP: α -Flag), and β C1-WT was analyzed using WB with an antibody to GFP (WB: α -GFP). (Middle three panels) Inputs of NbAS1-Flag, β C1-WT-GFP, and SnRK1-HA by WB. (Bottom) WB analysis was carried out using an antibody to actin for a loading control. (A and B) Experiments were repeated three times with similar results.

2D/2E). To investigate this, we amplified the *AS1* gene of *N. benthamiana* (NbAS1) and examined whether β C1-WT and its phosphorylation mimic mutants (β C1-2D and β C1-2D/2E) interact with NbAS1 using coimmunoprecipitation (CoIP) assays in *N. benthamiana* plants. *N. benthamiana* leaves were coinfiltrated with *Agrobacteria* capable of expressing NbAS1 and β C1-WT or its phosphorylation mimic mutants. Leaves were harvested 36 h after coinfiltration, and proteins were extracted and analyzed by CoIP. As shown in Fig. 9A, β C1-WT interacted strongly with NbAS1 in *N. benthamiana* leaves, whereas the interactions between NbAS1 and β C1-2D or β C1-2D/2E were greatly decreased. These data indicate that β C1-WT is associated with AS1 in tobacco plants and that phosphorylation mimics of TYLCCNB- β C1 weaken its interaction with AS1.

To further confirm the above-mentioned result and to determine the effect of phosphorylation of TYLCCNB- β C1 on its interaction with NbAS1, we introduced SnRK1, which is known to phosphorylate TYLCCNB- β C1 (27). Consistently, overexpression of SnRK1 abolished the interaction between TYLCCNB- β C1 and NbAS1 in a dose-independent manner in *N. benthamiana* leaves (Fig. 9B). Together, these results suggest that phosphorylation of TYLCCNB- β C1 largely abolishes its interaction with NbAS1 to attenuate virus infection symptoms in *N. benthamiana* plants.

DISCUSSION

Protein phosphorylation is a common and important posttranslational modification that can alter protein function via addition of phosphate groups to a target protein through the actions of various kinases (43). Geminivirus proteins, including the begomovirus NSP, movement protein, AC3, AC4, BC1, BV1 and capsid protein, the curtovirus C4 and AL2/C2 proteins, and the satellite β C1 protein, are also substrates of host

protein kinases during plant-virus interactions (27, 28, 44–49). It is well established that host Ser/Thr phosphorylation of plant viral proteins plays vital roles in attenuation of symptom development and efficiency of viral infection (28, 48–52). Consistent with this view, our previous work has shown that phosphorylation at Ser-33 and Thr-78 of TYLCCNB- β C1 by SnRK1 significantly attenuates its pathogenesis during Y10A/ β infection (27). Furthermore, overexpression of SnRK1 in *N. benthamiana* plants attenuates virus symptoms and decreases viral DNA accumulation during Y10A/ β infection, whereas knockdown of SnRK1 results in the reciprocal effects (27). However, little is known about the importance of Tyr phosphorylation of geminivirus proteins, with the exception that Tyr phosphorylation of a pomovirus MP impairs its cell-to-cell movement in plants (53). In this study, we identified two Tyr residues in TYLCCNB- β C1 that are phosphorylated by SnRK1 (Fig. 1) and found that a quadruple mutant of TYLCCNB- β C1 that mimics phosphorylation is defective as a pathogenicity determinant (Fig. 2B and 3B). Mimicking phosphorylation of TYLCCNB- β C1 also weakened its ability to reverse TGS (Fig. 6A) and to suppress PTGS (Fig. 7A) and abolished its interaction with NbAS1 (Fig. 9). Together with our previous data (27), these results suggest that phosphorylation of TYLCCNB- β C1 by SnRK1 is an important defense strategy against Y10A/ β infection. The results of our study significantly expand the scope of phosphorylation-mediated host defense against geminiviruses by implicating Tyr phosphorylation in the process.

Although phosphorylation of TYLCCNB- β C1 was reported to attenuate viral infection in our previous study (27), how the phosphorylation of TYLCCNB- β C1 impacts its pathogenic function remained to be investigated. The present study showed that a quadruple mutant that mimicked phosphorylation of TYLCCNB- β C1 does not induce disease symptoms associated with the betasatellite (Fig. 2B and 3B). Functional studies indicated that this phenomenon was not a result of decreased protein stability or altered subcellular localization of the TYLCCNB- β C1 phosphorylation mimics (Fig. 3, 4, and 5). Earlier studies showed that TYLCCNB- β C1 functions as a VSR to reverse methylation-mediated TGS (18) and to suppress PTGS (15–17). We therefore performed experiments to test whether phosphorylation of TYLCCNB- β C1 affected its ability to function as the VSR. We found that mimicking phosphorylation of TYLCCNB- β C1 impairs its function as a VSR at the level of both TGS and PTGS (Fig. 6 and 7). Furthermore, mimicking phosphorylation of TYLCCNB- β C1 weakened its ability to upregulate *Nbrgs-CaM*, an endogenous suppressor of RNA silencing (Fig. 8).

In addition to functioning as a VSR, TYLCCNB- β C1 acts as a symptom determinant (13, 14). In both *Nicotiana* and *Arabidopsis*, plants overexpressing TYLCCNB- β C1 display virus-like symptoms, such as leaf curling, petiole elongation, and twisted shoots (13, 14). In transgenic *Arabidopsis*, TYLCCNB- β C1 interacts with AS1 to alter leaf development, which largely phenocopies symptoms observed in virus-infected tobacco plants (14). More importantly, the results presented here, together with previous observations (27), clearly indicate that phosphorylation of TYLCCNB- β C1 is able to attenuate disease symptoms of TYLCCNB- β C1, which led us to speculate that phosphorylation of TYLCCNB- β C1 may affect its ability to function as a symptom determinant at the level of interacting with AS1. CoIP experiments showed that β C1-WT and NbAS1 interact *in vivo*, whereas the interactions between phosphorylation mimic mutants of TYLCCNB- β C1 and NbAS1 were greatly decreased (Fig. 9A). This suggests that mimicking phosphorylation of TYLCCNB- β C1 impairs its association with NbAS1. We also found that transient overexpression of SnRK1 abolished the interaction between β C1-WT and NbAS1 in a dose-independent manner in *N. benthamiana* leaves (Fig. 9B), in accordance with our previous observation that constitutive expression of SnRK1 in transgenic plants slows down the development of virus-induced symptoms (27). We interpret this to show that phosphorylation of TYLCCNB- β C1 negatively regulates symptom development as a consequence of an inability to interact with NbAS1. In fact, AS1 has been well characterized as a MYB domain transcription factor that regulates a set of genes involved in leaf development in a complex with the LATERAL ORGAN BOUNDARIES domain transcription factor AS2 in *Arabidopsis* (54, 55). AS1 has also been reported to be involved in immune responses via selective suppression of JA-responsive genes (14,

56, 57). The precise mechanisms by which a TYLCCNB- β C1-AS1 complex regulates leaf development and/or immune responses should be investigated in the future.

In conclusion, we have identified two novel Tyr phosphorylation sites of TYLCCNB- β C1 that appear to be targets for phosphorylation and have shown that phosphorylation mimic mutations of TYLCCNB- β C1 impaired its function as a pathogenicity determinant by weakening its ability to reverse TGS and to uppress PTGS and abolishing its interaction with NbAS1 in *N. benthamiana* leaves. To our knowledge, this is the first report establishing an inhibitory effect of phosphorylation of TYLCCNB- β C1 on its pathogenic functions as both a VSR and a symptom determinant and to provide a mechanistic explanation of how SnRK1 acts as a host defense factor. These findings expand the scope of phosphorylation-mediated defense and contribute to further understanding of plant defense mechanisms against geminiviruses.

MATERIALS AND METHODS

Plant material and growth conditions. Wild-type transgenic GFP 16c (58) and 16-TGS (37) *N. benthamiana* lines were used in this study. All experimental plants were grown in an insect-free chamber at 25°C with a 16/8-hour (light/dark) photoperiod. Plants at the 4- to 6-leaf stage were used for the experiments, as described previously (18).

Site-directed mutagenesis and plasmid construction. Site-directed mutagenesis of TYLCCNB- β C1 was performed using overlapping PCR as described previously (59). A Tyr residue at position 5 was changed to Phe (F) (TAC to TTT) or Glu (E) (TAC to GAA), a serine residue at position 33 was changed to Ala (A) (TCA to GCA) or Asp (D) (TCA to GAT), a Thr residue at position 78 was changed to Ala (A) (ACA to GCA) or Asp (D) (ACA to GAT), and a Tyr residue at position 110 was changed to Phe (F) (TAT to TTT) or Glu (E) (TAT GAA). Construction of infectious clones containing TYLCCNB- β C1 mutants was performed as described previously (12). To construct plasmids producing recombinant glutathione S-transferase (GST)-tagged proteins, the ORFs of β C1-WT and the mutants were cloned into the BamHI-XhoI sites of the pGEX-4T-3 vector (GE). The GST-GRIK and GST-SnRK1-KD constructs were described previously (27, 60). To generate the PVX expression constructs, ORFs of β C1-WT and its mutants were cloned into the AscI-SalI sites of the PVX vector pGR106 (34). For transient expression of WT and mutant β C1 proteins, ORFs were cloned into the KpnI-BamHI sites of the binary vector pCHF3 (61). Plasmids containing 35S:GFP and P19 constructs used for protein subcellular localization and the BiFC assay were constructed as previously described (16, 18, 35). For dual-luciferase assays, a 1.5-kb (relative to the ATG at bp +1) promoter of *Nbgs-CaM* was cloned into the XhoI-NcoI sites of pGreenII0800-LUC (62) to generate the P_{NbCaM} :LUC reporter construct. To determine phosphotyrosine in wild-type and mutant TYLCCNB- β C1 proteins, ORFs were cloned into the KpnI-BamHI sites of a binary vector, 35SGFP. For CoIP assays, ORFs of WT and mutant TYLCCNB- β C1 were cloned into the KpnI-BamHI sites of the binary vector 35SGFP, and ORFs of NbAS1 and SnRK1 were cloned into the SacI-BamHI sites of 35SFlag or the KpnI-BamHI sites of 35SHA binary vectors. All the primers used in these experiments are available upon request, and all constructs were confirmed by sequencing.

Recombinant protein production and kinase assay. Recombinant proteins were produced in *Escherichia coli* strain BL21(DE3) induced with 0.5 mM isopropyl β -D-thiogalactoside (IPTG) for 6 h at 20°C. GST-fused proteins were purified using glutathione resin (GE) according to the manufacturer's instructions. *In vitro* kinase assays were performed as described previously (27).

Viral inoculation and agroinfiltration. For inoculation and TGS experiments, *Agrobacterium tumefaciens* cultures carrying infectious clones were infiltrated into *N. benthamiana* leaves as described previously (18). For recombinant PVX vectors expressing WT or mutant TYLCCNB- β C1, each *A. tumefaciens* culture was adjusted to an optical density at 600 nm (OD_{600}) of 0.8 before infiltration into *N. benthamiana* plants. Transient silencing suppression assays were performed as described previously (16, 40). For protein subcellular localization, BiFC, dual-luciferase reporter assays, immunoprecipitation (IP), and CoIP, *A. tumefaciens* cultures were used at an OD_{600} of 1.0 unless otherwise stated.

Infectivity test and viral DNA accumulation. The course of viral infection was monitored as described previously (27, 33). Total nucleic acids were extracted from systemically infected leaves using a cetyltrimethylammonium bromide (CTAB)-based method (63). Viral DNA accumulation was measured using qPCR as described previously (26). Relative viral DNA accumulation levels were calculated by the comparative threshold cycle (C_T) method (64). The *N. benthamiana* 25S nuclear rRNA gene (*Nb25SrRNA*) was used as the endogenous control (65). The reactions were performed in triplicate, and the results were averaged.

RNA extraction and qPCR analysis. Total RNA was extracted from samples using an RNeasy Plant kit (Qiagen), and cDNA was reverse transcribed from 1.0 μ g of total RNA using a ReverTra Ace qPCR reverse transcription (RT) kit (Toyobo). Relative quantification of gene expression by qPCR was performed as described previously (66). Relative expression levels were calculated by the comparative C_T method (64). *NbACTIN2* RNA was used as the endogenous control. The reactions were performed in triplicate, and the results were averaged.

Protein extraction, WB analysis, IP, and CoIP. Protein extraction and WB analysis were performed as described previously (66). For IP and CoIP assays, 1.0 g of agroinfiltrated *N. benthamiana* leaf tissue was collected for each combination and homogenized in IP buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.6% Triton X-100, 10% glycerol, 5 mM dithiothreitol [DTT], 1 mM phenylmethylsulfonyl

fluoride [PMSF], 100 μ M MG132 with complete protease inhibitor cocktail [Roche]) and centrifuged twice at 13,000 rpm at 4°C for 15 min. The supernatant was incubated with 30 μ l protein G agarose (Millipore) and 1 μ g anti-Flag (α -Flag) antibody (Sigma) at 4°C for 4 h with gentle shaking and then washed six times with 1 ml of washing buffer (50 mM Tris-HCl, pH 7.5, 200 mM NaCl, 1 mM EDTA, 0.2% Triton X-100, 10% glycerol, 5 mM DTT, 1 mM PMSF). Immunoprecipitated proteins were analyzed by WB analysis using an anti-pY99 (Santa Cruz) or an α -GFP (Sigma) antibody.

Protein subcellular localization and BiFC assay. *N. benthamiana* leaves were infiltrated with *A. tumefaciens* cultures harboring the designated constructs, 0.5-cm² leaf explants were harvested approximately 48 h postinfiltration, and GFP fluorescence was examined by confocal microscopy as described previously (18). BiFC experiments were performed as described previously (18, 36). YFP fluorescence was observed and photographed 48 h postinfiltration using confocal microscopy. The nucleus was located using a nuclear-localized red fluorescent protein (RFP)-histone 2B (67).

Chop-PCR. Total nucleic acids were extracted from systemically infected leaves using a Hi-DNA secure plant kit (Tiangen). Chop-PCR was performed as described previously (39, 68). Briefly, 1 μ g total nucleic acids was digested with a methylation-sensitive restriction endonuclease, HinfI, or a methylation-dependent restriction endonuclease, McrBC (NEB), in a 20- μ l reaction mixture according to the manufacturer's recommendations. Undigested samples were treated in the same way but without adding the enzymes. After digestion, PCR was performed using 2 μ l of the digested DNA as the template in a 20- μ l reaction mixture and using the 35S promoter-specific primer pairs (35SP-F, 5'-AAGGYAAGTAATAGAGATTGGAG-3', and 35SP-R, 5'-CACCTTCCTTTCCACTATCTTCAC-3'), and the PCR products were separated by electrophoresis on a 1.5% agarose gel.

Dual-luciferase reporter assays. Transient dual-luciferase assays in *N. benthamiana* were performed as described previously (62, 69). LUC and REN were assayed by dual-luciferase assay (Promega) as previously described (70, 71). Six biological replicates were measured for each sample.

Statistical analysis. The data shown were calculated as the mean $\pm$ standard deviation (SD) for at least three independent experiments. Differences in the mean values were assessed using the statistical software data processing system (DPS) v7.05 (72), followed by Tukey's test. Values were considered significantly different at a *P* value of <0.05.

ACKNOWLEDGMENTS

This work was supported by grants from the National Natural Science Foundation of China (31390422) and the Postdoctoral Science Foundation of China (2015M581946).

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