Tomato SI\(\text{SnRK1}\) Protein Interacts with and Phosphorylates \(\beta\)C1, a Pathogenesis Protein Encoded by a Geminivirus \(\beta\)-Satellite\(^1\)[W][OA]

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The \(\beta\)C1 protein of tomato yellow leaf curl China \(\beta\)-satellite functions as a pathogenicity determinant. To better understand the molecular basis of \(\beta\)C1 in pathogenicity, a yeast two-hybrid screen of a tomato \((\text{Solanum lycopersicum})\) cDNA library was carried out using \(\beta\)C1 as bait. \(\beta\)C1 interacted with a tomato \(\text{SUCROSE-NONFERMENTING1-related kinase designated as SI\(\text{SnRK1}\)}). Their interaction was confirmed using a bimolecular fluorescence complementation assay in \(\text{Nicotiana benthamiana}\) cells. Plants overexpressing \(\text{SnRK1}\) were delayed for symptom appearance and contained lower levels of viral and satellite DNA, while plants silenced for \(\text{SnRK1}\) expression developed symptoms earlier and accumulated higher levels of viral DNA. In vitro kinase assays showed that \(\beta\)C1 is phosphorylated by SI\(\text{SnRK1}\) mainly on serine at position 33 and threonine at position 78. Plants infected with \(\beta\)C1 mutants containing phosphorylation-mimic aspartate residues in place of serine-33 and/or threonine-78 displayed delayed and attenuated symptoms and accumulated lower levels of viral DNA, while plants infected with phosphorylation-negative alanine mutants contained higher levels of viral DNA. These results suggested that the SI\(\text{SnRK1}\) protein attenuates geminivirus infection by interacting with and phosphorylating the \(\beta\)C1 protein.

In nature, plants are continuously exposed to attacks by a variety of microbial pathogens, including viruses. To combat attack, plants have evolved complex mechanisms to respond to virus challenge, including the hypersensitive response mediated by resistance genes and posttranscriptional gene silencing (Whitham et al., 1994; Vanitharani et al., 2005). The elucidation of host-virus interactions will be important for providing additional clues to basic compatibility functions as well as host surveillance mechanisms.

Geminiviruses are a group of plant DNA viruses characterized by single-stranded circular genomes encapsidated in twinned icosahedral particles that range in size from 18 to 30 nm (Hanley-Bowdoin et al., 2000; Rojas et al., 2005). They can be divided into four genera (\(\text{Mastrevirus}\), \(\text{Topocuvirus}\), \(\text{Curtovirus}\), and \(\text{Begomovirus}\)) based on genome structure, insect vectors, and host range (Fauquet and Stanley, 2005). Within the family Geminiviridae, begomoviruses are the most numerous and geographically widespread viruses. Begomoviruses have either monopartite or bipartite genomes (Hanley-Bowdoin et al., 2000; Fauquet et al., 2003). In recent years, a \(\beta\)-satellite molecule, which is a circular single-stranded DNA of approximately 1,350 nucleotides, has been associated with some monopartite begomoviruses, where it is essential for the induction of typical disease symptoms (Saunders et al., 2000, 2003; Bridgon et al., 2001; Jose and Usha, 2003; Cui et al., 2004). Full-length \(\beta\)-satellite molecules encode an approximately 13.5-kD protein known as \(\beta\)C1 on the complementary sense strand. \(\beta\)C1 is a pathogenicity determinant and a suppressor of RNA silencing (Jose and Usha, 2003; Cui et al., 2004, 2005; Saunders et al., 2004; Qian and Zhou, 2005; Saeed et al., 2005; Gopal et al., 2007; Kon et al., 2007). Previous studies showed that \(\beta\)C1 interacts with \(\text{ASYMMETRIC LEAVES1}\) to alter leaf development and suppress selected jasmonic acid responses (Yang et al., 2008). \(\beta\)C1 also interacts with a host ubiquitin-conjugating enzyme, SI\(\text{UBC3}\), and modifies the host ubiquitination system (Eini et al., 2009). Here, we show that \(\beta\)C1 of the tomato yellow leaf curl China \(\beta\)-satellite (TYLCCNB) interacts with a tomato...
(Solanum lycopersicum) SUCROSE-NONFERMENTING1 (SNF1)-related kinase (SlSnRK1). We also demonstrate that SlSnRK1 phosphorylates the βC1 protein in vitro and that mutations in the primary phospho residues impact the pathogenicity function of βC1.

RESULTS

βC1 Interacts with Tomato SlSnRK1

To identify host proteins that interact with the βC1 protein of TYLCCNB (TYLCNB-βC1), we performed a yeast two-hybrid screen of a tomato cDNA library fused to the GAL4 activation domain using TYLCCNB-βC1 fused to the GAL4 DNA-binding domain as bait. From a total of 5 × 10⁵ independent, double transformants assayed for His prototrophy and α-galactosidase activity, one cDNA clone that interacts with βC1 was identified. The clone displayed 100% sequence identity with a cDNA encoding a tomato SNF1-related kinase (AF143743; Bradford et al., 2003), which we have named SlSnRK1. Based on the complete nucleotide sequence of AF143743, the full-length coding sequence of SlSnRK1 was amplified from tomato cDNA using primers SlSnRK1-1F-Ec and SlSnRK1-1R-Ba, and its interaction with βC1 was confirmed by the yeast two-hybrid system (Fig. 1A).

The full-length SlSnRK1 cDNA is 1,545 nucleotides and encodes a protein of 514 amino acids (58,824 D). It is closely related to SlSnRK1 in Nicotiana benthamiana, NPK5 in tobacco (Nicotiana tabacum), and AKIN11 in Arabidopsis (Arabidopsis thaliana), with identities of 95%, 87%, and 78%, respectively (Fig. 1B; Supplemental Fig. S1). As a typical member of the SnRK1 subfamily, SlSnRK1 encodes the α-subunit of the SnRK1 heterotrimer, which is a key regulator of the plant response to starvation and metabolic stress (Bradford et al., 2003; Halford and Hey, 2009). SlSnRK1 contains a conserved kinase domain (KD) in the N terminus, an internal ubiquitin-associated domain (UBA), and an autoinhibitory sequence (AIS) domain, as well as a C-terminal domain (CTD) that is responsible for α-subunit binding and formation of the SnRK1 complex (Fig. 1C).

βC1 and SlSnRK1 Interaction in Planta

Bimolecular fluorescence complementation (BiFC) was performed in agro-infiltrated N. benthamiana leaves to test for interaction between βC1 and SlSnRK1 in plant cells. For this assay, βC1 was fused to the N-terminal fragment of yellow fluorescent protein (YFPN; pβC1-YFPN) and SlSnRK1 was fused to the C-terminal fragment of YFP (YFPC; pSlSnRK1-YFPC). Pair-wise expression of pβC1-YFPN and pSlSnRK1-YFPC resulted in a YFP fluorescence signal in the cytoplasm of agroinfiltrated cells at 72 h post infiltration, but no YFP fluorescence was observed when pβC1-YFPN and pYFPF, or pSlSnRK1-YFPC and pYFPF, were coexpressed (Fig. 2). These results confirm that the βC1 protein interacts with SlSnRK1 in plant cells.

Subcellular Localization and Expression Pattern of SlSnRK1

Subcellular localization of GFP-tagged SlSnRK1 was examined by agroinfiltration of N. benthamiana epidermal cells (Fig. 3). Green fluorescence was detected in the cytoplasm and nuclei of cells expressing GFP-SlSnRK1. A similar fluorescence pattern was observed in cells expressing the GFP control protein. These data are consistent with the localization of GFP-tagged SlSnRK1 to both the cytoplasmic and nuclear compartments of plant cells.

To determine the expression pattern of SlSnRK1 mRNA, quantitative reverse transcription (RT)-PCR was performed using total RNA from various tomato tissues as template. The highest level of SlSnRK1 mRNA was detected in the flower, with intermediate levels in the leaf and the lowest levels in the root and stem (Fig. 4A). We also compared SlSnRK1 transcript
levels in tomato leaves inoculated with TYLCCNV/TYLCCNB or with TYLCCNV alone by quantitative RT-PCR. Higher levels of SlSnRK1 mRNA were also detected in TYLCCNV/TYLCCNB-infected than in TYLCCNV-infected leaves at 3 d post inoculation (DPI; Fig. 4B). These results suggested that TYLCCNB stimulates the accumulation of the SlSnRK1 mRNA.

Identification of the Domains Necessary for βC1 and SlSnRK1 Interaction

To locate the domains necessary for βC1 and SlSnRK1 interaction, we tested eight deletion mutants for βC1 and nine deletion mutants for SlSnRK1, respectively (Fig. 5) in yeast two-hybrid assays. As shown in Figure 5A, yeast transformants harboring SlSnRK1 mutants M1 (amino acids 281–514), M2 (amino acids 1–339), M4 (amino acids 1–460), M5 (amino acids 1–360 fused with 450–514), or M6 (amino acids 1–290 fused with 339–514) grew on TDO/Aba+ (for synthetic dextrose/His/Leu/Trp medium in the presence of 90 or 120 ng mL−1 aureobasidin A) plates, indicative of interaction with βC1. In contrast, mutant M3 (amino acids 1–280) failed to interact with βC1. No βC1-binding activity was detected for the kinase domain alone (residues 1–280), and deletion of the kinase domain did not affect the interaction between SlSnRK1 and βC1. Based on these results, we concluded that the kinase domain is not involved in βC1 binding. Instead, our data suggested that the central and C-terminal regions are involved in binding βC1.

To further define the βC1-binding domain, we constructed three mutants (M7–M9) that divided SlSnRK1-M1 into known functional domains. Yeast two-hybrid assay revealed that both M7 (residues 281–339, containing the UBA domain) and M8 (residues 340–449, containing the AIS domain) retained significant βC1-binding activity, while M9 (residues 450–514, containing the CTD) did not show any binding activity (Fig. 5A). These results suggested that the UBA and AIS domains in SlSnRK1 can each interact with βC1.

All of the βC1 mutants including deletions of only 10 amino acids of either the N or C terminus failed to interact with full-length SlSnRK1 (Fig. 5B), indicating that full-length βC1 is required for interaction with SlSnRK1.

Plant SnRK1 Affects TYLCCNV/TYLCCNB Infection and Alters Viral DNA Accumulation

To assess the biological significance of SlSnRK1-βC1 interactions in vivo, we inoculated wild-type and...
transgenic *N. benthamiana* plants carrying an Arabidopsis antisense SnRK1 expression cassette (AS-12) or an Arabidopsis sense SnRK1 expression cassette (S-5; Hao et al., 2003) with TYLCCNV/TYLCCNB and monitored infection over time (Carvalho et al., 2008b). Changes in SnRK1 expression altered the timing of symptom appearance, with overexpressing plants developing symptoms later and silenced plants showing symptoms earlier than wild-type plants (Fig. 6A). The shift in the timing of symptom development was readily apparent when the infectivity data were expressed as days post inoculation to reach 50% of symptomatic plants (DPI 50%), with values of 7.7, 5.8, and 5.1 DPI 50% for SnRK1-overexpressing, silenced, and wild-type plants, respectively (Fig. 6B). However, both wild-type and transgenic plants displayed similar symptoms during the late stage of infection. DNA gel-blot analysis showed that SnRK1-overexpressing plants accumulated less viral DNA and that SnRK1-silenced plants accumulated more viral DNA when compared with wild-type plants infected with TYLCCNV/TYLCCNB (Fig. 6C). Together, these results suggested that increasing SnRK1 levels reduce infection efficiency while lowering SnRK1 levels enhances efficiency. To rule out the possible functional differences between Arabidopsis SnRK1 (AKIN11) and SlSnRK1, we showed that βC1 also interacts with AKIN11 (Supplemental Fig. S2).

**Impacts of SlSnRK1 Kinase Activity by βC1**

We next asked if βC1 can impact SlSnRK1 kinase activity in yeast cells. In initial experiments, we tested whether SlSnRK1 can complement the yeast *snf1* deletion strain Δsnf1 BY4741, which cannot grow on medium containing a carbon source other than Glc. Expression of SlSnRK1 in Δsnf1 BY4741 restored growth on synthetic complete medium containing 2% (w/v) Gal and 2% (w/v) Suc as carbon sources, but Δsnf1 BY4741 transformed with the empty expression plasmid pESC-Ura or a plasmid expressing the kinase-dead mutant, CTU-SlSnRK1 K48R, failed to grow on the medium (Fig. 7A). Thus, SlSnRK1 can functionally complement SNF1 in yeast and SlSnRK1 kinase activity is essential for complementation. We found that Δsnf1 BY4741 cotransformed with SCU-SlSnRK1 and SCL-βC1 grew similarly as Δsnf1 BY4741 cotransformed SCU-SlSnRK1 and pESC-Leu (positive control) on medium containing Gal and Suc as carbon sources, but Δsnf1 BY4741 cotransformed with pESC-Ura and SCL-βC1 (negative control) could not grow on the medium (Fig. 7B), suggesting that βC1 cannot inhibit SlSnRK1 activity.

**SlSnRK1 Phosphorylates βC1 Mainly on Thr at Position 78 and Ser at Position 33 in Vitro**

To test if SlSnRK1 phosphorylates βC1 protein, both proteins were expressed in *Escherichia coli* as glutathione S-transferase (GST) fusions. The SAMS peptide, HMRSAMSGLHLVKRR, which is a specific and sensitive substrate for the SNF1/AMPK/SnRK1 kinases (Sugden et al., 1999), was also fused to GST and used as a negative control, while GST alone was used as a positive control. The Arabidopsis GRIK1 protein has been reported to act as an upstream activating kinase of SnRK1 by phosphorylating its activation loop (Shen et al., 2009), so we used GST-tagged GRIK1 protein to activate SlSnRK1 during the in vitro kinase reactions. Because the full-length SlSnRK1 protein was insoluble, a truncated SlSnRK1 containing the KD and UBA domain (SlSnRK1-KD) was used for the in vitro phosphorylation assays. Recombinant βC1, SAMS, and GST proteins were separately incubated with GRIK1 and SlSnRK1-KD proteins under the same reaction conditions. Phosphorylation of βC1 was clearly ob-

**Figure 3.** Subcellular localization of SlSnRK1 in *N. benthamiana* epidermal cells. Micrographs showing cells expressing GFP (top row) or GFP-SlSnRK1 (bottom row) were examined under fluorescence (left), bright field (middle), or an overlay of bright and fluorescence illumination (right) by confocal microscopy. Arrows indicate nuclei. Bars = 50 μm.
Served after coincubation with SlSnRK1-KD, whereas GST was not phosphorylated (Fig. 8A), indicating that βC1 is a SlSnRK1-KD substrate in vitro. To rule out the possibility that phosphorylation of βC1 was catalyzed by GRIK1, an additional reaction containing GRIK1 and βC1 in the absence of SlSnRK1-KD was carried out. Autoradiography only detected a radiolabeled band for GRIK1, indicating that the viral protein βC1 is specifically phosphorylated by SlSnRK1.

To determine the βC1 residue(s) phosphorylated by SlSnRK1 in vitro, we first analyzed the coding sequence of full-length βC1 using NetPhos 2.0 for potential phosphorylation sites (http://www.cbs.dtu.dk/services/NetPhos/). The analysis revealed that the Thr at position 78 (Thr-78) and the Ser at position 33 (Ser-33) are potential phosphorylation sites for Ser/Thr kinases (data not shown). We generated six βC1 point mutants, βC1S33A, βC1S33D, βC1T78A, βC1T78D, βC1S33A/T78A, and βC1S33D/T78D, in which Ser-33 or Thr-78 of βC1 was individually or simultaneously replaced by Ala to eliminate phosphorylation

Figure 4. SlSnRK1 mRNA levels in various tomato tissues (A) or TYLCCNV/TYLCCNB- and TYLCCNV-infected plants at 3 DPI (B). Relative mRNA levels in tomato tissues were normalized using EF-1α mRNA as a reference. Values are means of three independent experiments. Different lowercase letters above the bars denote significant differences (Fisher’s LSD method; P < 0.05).

Figure 5. Identification of the binding domains responsible for the SlSnRK1-TYLCCNB-βC1 interaction. A, Schematic representation of the truncated mutants of SlSnRK1 and yeast two-hybrid analysis of their interactions with βC1. The yellow box represents the KD, the gray box represents the UBA domain, the light blue box represents the AIS, and the red box represents the CTD. B, Diagram of deletion mutants of βC1 used to determine the binding requirements for SlSnRK1. The Ser residue at position 33 is indicated as a dark blue box and the Thr residue at position 78 is labeled as a green box. [See online article for color version of this figure.]
or by Asp to mimic constitutive phosphorylation (Waigmann et al., 2000; Karger et al., 2003; Trutnyeva et al., 2005). The mutant proteins were expressed in E. coli as GST fusion proteins and used in kinase assays. As shown in Figure 8, B and C, mutations S33A, S33D, T78A, T78D, S33A/T78A, and S33D/T78D significantly reduced the level of bC1 phosphorylation relative to the wild-type protein. These differences were not due to different loading amounts of bC1 and its mutants (Fig. 8B). The reductions in radioactive signals for bC1S33A and bC1T78D were stronger than for bC1T78A and bC1T78D. These results indicated that both bC1 Ser-33 and Thr-78 are SlSnRK1 phosphorylation sites. Replacement of Ser-33 or Thr-78 with Asp did not promote bC phosphorylation of the other site, indicating that the two sites are unlikely to be synergistic for phosphorylation. Both double mutants bC1S33A/T78A and bC1S33D/T78D retained low but measurable phosphorylation signals (37%–28% of the wild-type bC1; Fig. 8C), suggesting that other bC1 residues might also be phosphorylated by SlSnRK1 in vitro.

Effect of bC1 Phosphorylation Site Mutations on Virus Infection and Viral DNA Accumulation

To assess the role of bC1 phosphorylation by SlSnRK1, Ser-33 and/or Thr-78 mutations were introduced into the bC1 gene of a satellite replicon. Six mutant constructs were generated, including single replacements at Ser-33 or Thr-78 to Ala (S33A and T78A) or Asp (S33D and T78D) as well as double mutants (S33A/T78A and S33D/T78D). Infectious clones of these mutants and wild-type TYLCCNB were used to inoculate N. benthamiana plants together with TYLCCNV. Delay of virus infection associated with mild symptoms was observed in plants inoculated with the Asp mutants TYLCCNB-S33D, TYLCCNB-T78D, and TYLCCNB-S33D/T78D (Fig. 9, A and B). When the infectivity data were expressed as DPI 50%,
the βC1 phosphomimic mutations (S33D, T78D, and S33D/T78D) reduced the efficiency of virus infection (Fig. 9C). DNA gel-blot analysis showed that viral DNA accumulation was lower in plants coinoculated with TYLCCNV and a βC1 phosphomimetic mutant (S33D, T78D, or S33D/T78D) than in plants coinoculated with TYLCCNV and wild-type TYLCCNB (Fig. 9D). In contrast, symptom appearance and severity associated with the TYLCCNB Al mutants (S33A, T78A, S33A/T78A) resembled wild-type TYLCCNB (Fig. 9, A and B), but viral DNA levels were higher in plants infected by the Al mutants versus the wild-type (Fig. 9D). These results indicated that mutations in the primary residues phosphorylated by SnRK1 in vitro impact the pathogenicity function of βC1.

DISCUSSION

Geminiviruses infect a broad variety of plants and induce a wide range of symptoms (Hanley-Bowdoin et al., 2000). Recent studies indicated that they can evade the plant immune system by interfering with host antiviral pathways (Hao et al., 2003; Wang et al., 2003; Florentino et al., 2006; Piroux et al., 2007). In response, the plant hosts have evolved diverse innate defense mechanisms to counter these challenges (Voinnet, 2001; Xie and Guo, 2006). Many host factors have been shown to be hijacked or coopted by geminiviruses to facilitate infection (Kong et al., 2000; Egelkrot et al., 2001; Carvalho et al., 2008a). In contrast, few host factors have been shown to participate in plant antiviral processes. For example, interaction between the RepA protein of Wheat dwarf virus (WDV) and a wheat NAC domain protein (GRAB) severely impairs WDV replication in cultured wheat (Triticum aestivum) cells (Xie et al., 1999). Similarly, the expression of sense RNAs of tomato SUMO (LeSUMO) impairs tomato golden mosaic virus (TGMV) replication, suggesting that plant SUMO may also play an important role in the plant antiviral defense response (Castillo et al., 2004).

TYLCCNV is a monopartite begomovirus associated with a β-satellite (TYLCCNB) identified in China (Zhou et al., 2003). We demonstrated previously that TYLCCNV alone produces asymptomatic infections in tobacco, tomato, and petunia (Petunia hybrida) and that TYLCCNB is required for the production of leaf curl symptoms in these hosts (Cui et al., 2004). These studies showed that the βC1 protein encoded by TYLCCNB is a pathogenicity factor that is necessary for symptom production. In this study, we showed that TYLCCNB-βC1 interacts with a tomato SNF1-related kinase designated as SLSnRK1 by yeast two-hybrid analysis and BiFC assay.

Yeast SNF1, mammalian AMPK, and plant SnRK1 are a group of Ser/Thr protein kinases that are conserved in all eukaryotes and have similar subunit compositions, subunit structures, and common kinase cascades (Hardie et al., 1998; Halford et al., 2003, 2004; Hardie, 2007; Polge and Thomas, 2007; Baena-González and Sheen, 2008). In plants, SnRK1 is widely recognized to be involved in various physiological processes, including nutrient and energy sensing, global regulation of metabolism, control of the cell cycle, modulation of development, and response to abiotic or biotic stress (Baena-González et al., 2007). Although a number of SnRK1 functions have been characterized, information about SnRK1 function against pathogen infection is limited. Hao et al. (2003) showed that the AL2 protein from TGMV (genus Begomovirus) and the L2 protein from Beet curly top virus (genus Curtovirus) interact with Arabidopsis SnRK1 (AKIN1) and that AL2 and L2 inactivate SnRK1, leading to enhanced susceptibility. These results suggested that metabolic alterations mediated by SnRK1 may contribute to plant innate antiviral defenses and that SnRK1 inactivation by AL2 and L2 is a counterdefense measure. Although βC1 also binds to SnRK1, we found that βC1 does not inhibit SnRK1 kinase activity and, instead, is phosphorylated by SLSnRK1. The βC1 phosphomimic mutants S33D and T78D attenuate symptoms, delay viral infection,
and reduce viral DNA accumulation, while the Ala mutants S33A and T78A enhance viral DNA accumulation, suggesting that phosphorylation of the βC1 protein negatively impacts its function as a pathogenicity determinant.

A number of viral nonstructural proteins are phosphoproteins and are phosphorylated by various plant kinases. Coat proteins (CPs) of the Cauliflower mosaic virus (Martinez-Izquierdo and Hohn, 1987) and potyviruses (Ivanov et al., 2001; Fernández-Fernández et al., 2002) as well as movement protein (MP) of Tobacco mosaic virus (Atkins et al., 1991; Watanabe et al., 1992; Citovsky et al., 1993; Waigmann et al., 2000) are phosphorylated, and several functions of these proteins are affected by phosphorylation (Karpova et al., 1999; Kawakami et al., 1999; Waigmann et al., 2000). Previous studies also identified a PERK-Like Receptor Kinase, NsAK, that may regulate nuclear shuttle protein function through phosphorylation (Florentino et al., 2006).

Protein phosphorylation may be a common process in response to virus challenge by plants. Our data support a model in which phosphorylation of βC1 by SIrSnRK1 is a counterdefense response against virus infection by the host. We demonstrated previously that transgenic N. benthamiana, tobacco, and Arabidopsis plants expressing the TYYCNCB βC1 gene are stunted and show leaf cupping and curling. The resulting “symptoms” are much more severe than those associated with TYYCCNV plus TYYCNCB infection, demonstrating that βC1 is very toxic to plants (Cui et al., 2004; Yang et al., 2008). SnRK1 phosphorylation of βC1 may be used by plants to overcome its detrimental effects. This idea is supported by our observation that reducing SnRK1 expression enhances the efficiency of TYYCCNV plus TYYCNCB infection and increases viral DNA accumulation.

An unanswered question is how the phosphorylation of βC1 impacts its pathogenicity function. We showed previously that the βC1 protein binds to DNA in a sequence-nonspecific manner, functions as a suppressor of RNA silencing, and is a pathogenicity protein that plays a vital role in symptom induction by suppression of the silencing defenses in plants (Cui et al., 2005). Phosphorylation of proteins can regulate their nucleic acid-binding properties (Boyle et al., 1991; Mayrand et al., 1993) and interactions between viral RNA and replication proteins of positive-strand RNA viruses (Shapka et al., 2005; Stork et al., 2005). Phosphorylation of βC1 may inhibit its ability to bind nucleic acid, which may negatively impact βC1 function as a RNA-silencing suppressor and result in attenuating viral infection. Alternatively, the stability of βC1 protein could be influenced by its phosphorylation status. Previous studies demonstrated that phosphorylation of hepatitis C virus NS5A (Pietzschmann et al., 2001), turnip yellow mosaic virus 66K protein (Héricourt et al., 2000; Jakubiec et al., 2006), or tobamovirus MP (Kawakami et al., 1999) can affect viral protein

![Figure 8](https://example.com/figure8.png)

**Figure 8.** In vitro phosphorylation of βC1. A, SIrSnRK1 can specifically phosphorylate βC1. Coomassie blue-stained SDS-PAGE gels (12%; top panel) and the corresponding autoradiograph images (bottom panel) are shown. Due to the similar molecular masses, GST-SAMS (approximately 27 kD) comigrates with GST (approximately 26 kD) during electrophoresis. B, SIrSnRK1 phosphorylates βC1 primarily at Ser-33 and Thr-78. The asterisk represents GST contaminants during purification, and no phosphorylation signal was detected on them. C, The radioactive signals shown in B were quantified by ImageQuant TL V2003 software. All data represent means ± SE of three replicate experiments. Different lowercase letters above the bars denote significant differences (Fisher’s LSD method; *P* < 0.05).
stability. In addition, protein phosphorylation often plays a role in ubiquitin-mediated proteolysis, and SCF (one type of multisubunit ubiquitin-protein ligase [E3]) degradation pathways are mediated by phosphorylation-dependent substrate recognition (Kong and Chock, 1992; Clurman et al., 1996; Won and Reed, 1996; Musti et al., 1997; Willems et al., 1999; Pickart, 2001; Feng et al., 2004; Gao et al., 2004; Dreher and Callis, 2007). The recent discovery that \( \beta C1 \) protein is degraded by the 26S proteasome (Yang et al., 2008) indicates that SlSnRK1 may interact with and phosphorylate \( \beta C1 \) for degradation by the 26S proteasome, leading to the attenuation of symptoms and reduction of the efficiency of viral infection.

In conclusion, we have demonstrated that tomato SlSnRK1 protein interacts with and phosphorylates \( \beta C1 \), a pathogenicity factor encoded by a geminivirus \( \beta \)-satellite. Future studies will determine whether the phosphorylation of \( \beta C1 \) negatively impacts its function as a RNA-silencing suppressor and/or mediates its degradation by the 26S proteasome.

Figure 9. Effects of \( \beta C1 \) phosphorylation site mutations on virus infection and relative viral DNA accumulation levels in agroinoculated *N. benthamiana* plants. A, Symptoms of plants agroinoculated with TYCCNV and TYCCNB or its mutants (TYLCCNB-S33A, TYLCCNB-S33D, TYLCCNB-T78A, TYLCCNB-T78D, TYLCCNB-S33A/T78A, and TYLCCNB-S33D/T78D) at 17 DPI. CK- indicates the mock-inoculated plant. B, Infection course of TYCCNV and TYCCNB or its mutants. Values represent percentages of systemically infected plants at different DPI and are given as means ± SD of triplicate experiments. In each experiment, 12 plants were inoculated. C, DPI 50% after inoculation of TYLCCNV and TYLCCNB or its mutants. The data are means ± SD of triplicate experiments. Different lowercase letters above the bars denote significant differences (Fisher’s LSD method; \( P < 0.05 \)). D, TYLCCNV and TYCCNB DNA levels in wild-type and transgenic plants expressing antisense SnRK1 (AS-12) or sense SnRK1 (S-5) at 8 and 15 DPI. After infection, total DNA from a whole-plant mixture was used for DNA gel blotting. Blots were probed with the CP gene sequence of TYLCCNV (top) or the full-length sequence of TYLCCNB (middle). An ethidium bromide-stained gel shown below the blots provides a DNA-loading control. The positions of single-stranded (ssDNA) and subgenomic (sgDNA) forms of TYLCCNV and TYLCCNB are indicated.
MATERIALS AND METHODS

Plant Material, Growth Conditions, and Genotyping

Tomato (Solanum lycopersicum ‘Hongbaoshi’) was used to construct the tomato cDNA library. Antisense (AS-12) and sense (S-9) AKIN11 transgenic Nicotiana benthamiana lines (Hao et al., 2003) were kindly provided by Dr. David M. Bisaro. Plants were grown in 10-cm pots filled with a mixture of 60% vermiculite and 40% meadow soil in a growth chamber at 25°C under long-day conditions (16 h of light/h of dark).

Plasmid Construction

The plasmids used in this study are listed in Supplemental Table S1. The primers used for mutagenesis and subcloning are given in Supplemental Table S2.

To produce plasmids for yeast two-hybrid screen and tox, the coding sequence of the full-length βC1 protein and eight deletion fragments containing four N-terminal deletion mutants, βC1-M1 (residues 40–118), βC1-M4 (residues 11–118), βC1-M5 (residues 21–118), and βC1-M6 (residues 31–118), as well as four C-terminal deletion mutants, βC1-M2 (residues 1–73), βC1-M3 (residues 1–39), βC1-M7 (residues 1–91), and βC1-M8 (residues 1–101), were amplified separately using the primer pairs listed in Supplemental Table S2, with the downstream repeat construct of TYLCCNB in pBI-Nplus (Cui et al., 2004) as the template. The PCR fragments were inserted into the EcoRI-BamHI sites of the yeast GAL4 binding domain vector pGBKTK or GAL4 activation domain vector pGADT7 (Clontech), resulting in the recombinant plasmids listed in Supplemental Table S1.

The coding sequence of intact SlSnRK1 was amplified from a tomato leaf cDNA at the six-leaf stage with primer pair SlSnRK1-1F-Ec/SlSnRK1-1R-Ba, and seven deletion mutant fragments of SlSnRK1 containing N-terminal truncated mutant SlSnRK1-M1 (residues 281–514), C-terminal truncated mutants SlSnRK1-M2 (residues 1–339), SlSnRK1-M3 (residues 1–280), and SlSnRK1-M4 (residues 1–460), and central mutants SlSnRK1-M7 (residues 281–339), SlSnRK1-M8 (residues 340–449), and SlSnRK1-M9 (residues 450–514) were separately amplified with the primers listed in Supplemental Table S2. Overlap extension PCR was used to generate the two internal deletion mutants SlSnRK1-M5 (residues 1–514 with deletion of amino acids 361–461, which removes the potential motif corresponding to the autoinhibitory sequence in mammalian AMPK or yeast Snf1) and SlSnRK1-M6 (residues 1–514 with deletion of amino acids 291–339, which removes the putative UBA; Tao et al., 2002). All of the amplified products were inserted into the EcoRI-BamHI site of the vector pGBKTK7 or vector pGADT7 (Clontech), resulting in the recombinant plasmids listed in Supplemental Table S1.

The coding sequences of the full-length Arabidopsis (Arabidopsis thaliana) AKIN11 were amplified separately using the primer pairs listed in Supplemental Table S2. The AKIN11 PCR fragments were digested with EcoRI and BamHI and cloned into the vector pGBKTK7 and vector pGADT7 (Clontech) to generate the recombinant plasmids listed in Supplemental Table S1.

For the production of BiFC vectors, the full-length coding sequence of tomato SlSnRK1 was amplified using the primers listed in Supplemental Table S2. The PCR fragments were digested with KpnI and BamHI and cloned into the vector pY2C-CT-Ura (Invitrogen), resulting in the recombinant plasmids listed in Supplemental Table S1.

For subcellular localization studies, SlSnRK1 was tagged at its N terminus with GFP by inserting PCR-amplified SlSnRK1 cDNAs using the primer pair listed in Supplemental Table S2 into the BamHI-PstI site of pCH3-GFP to produce GFP-SlSnRK1.

For yeast complementation studies, the full-length coding sequence of tomato SlSnRK1 was amplified using the primer pair listed in Supplemental Table S2 and cloned into the EcoRI-SphI site of pYES-Ura (2μ origin, URA3 selection marker; Strategene) or the KpnI-BamHI site of pRC2/CT-Ura (CEN6/ARS4 origin, URA3 selection marker; Invitrogen), resulting in SUC1-SlSnRK1 or CTU- SlSnRK1, respectively. The dead kinase mutant SlSnRK1K48R was generated by overlapping PCR (Tao et al., 2002) using the complementary primer pairs listed in Supplemental Table S2.

For the construction of infectious clones of TYLCCNB-bC1 mutants, site-directed mutagenesis was performed to alter βC1 Ser-33 coding triplet TCA to Ala (A) coding triplet GCA or Asp (D) coding triplet GAC, or both Ser-33 and Thr-78 to Ala (A) or Asp (D), resulting in mutants βC1S33A, βC1T78A, βC1S33D, βC1T78D, βC1S33A/T78A, and βC1S33D/T78D. The TYLCCNB infectious clones harboring corresponding βC1 mutants were named TYLCCNB-S33A, TYLCCNB-S33D, TYLCCNB-T78A, TYLCCNB-T78D, TYLCCNB-S33A/T78A, and TYLCCNB-S33D/T78D, respectively. The single base mutations were generated by the overlapping PCR (Tao et al., 2002) using the complementary primer pairs listed in Supplemental Table S2. The overlapping PCR products were inserted into the pGEM-T Easy (Promega) vector to produce clones pGEMβC1S33A, pGEMβC1T78A, pGEMβC1S33D, pGEMβC1T78D, pGEMβC1S33A/T78A, and pGEMβC1S33D/T78D. The double mutation pGEMβC1S33D/T78D and pGEMβC1S33D were generated by overlapping PCR (Tao et al., 2002) with complementary primer pairs Y10βC1S33A/F-Y10βC1T78A-R and Y10βC1S33D/F-Y10βC1S33D-R using pGEMβC1T78D and pGEMβC1S33A/T78A as the template, respectively. The fidelity of the mutants was confirmed by sequencing. The strategy described previously (Zhou et al., 2003) was then used for the construction of infectious clones (Supplemental Table S1) of βC1 mutants.

The plasmid pNSB1554 harboring GST-fused GRIK1 was constructed previously (Shen et al., 2009). The plasmid pGEX-KS-SAMS expressing the positive control peptide SAMS for kinase assay was kindly provided by Dr. David M. Bisaro.

To generate the GST-SlSnRK1-KD expression vector, the plasmid pGADT7-SlSnRK1-M2 was digested with EcoRI and Xhol and then inserted into pGEX-4T-1 vector (GE Healthcare). To construct the GST-tagged TYLCCNB-bC1 for in vitro kinase assay, pGEM6, pGEMβC1S33A, pGEMβC1T78A, pGEMβC1S33D, pGEMβC1T78D, or pGEMβC1S33D/T78D was used as the PCR template with primer pair Y10βC1S33A-F/Y10βC1T78A-R. The amplified fragments were inserted into the EcoRI-Xhol site of the vector pGEX-6P-1 (GE Healthcare).

Yeast Two-Hybrid Screens

The construction and screening of the tomato cDNA library and the analyses of positive interactions were performed according to the BD Matchmaker Library Construction and Screening Kits User Manual (Clontech). Total RNAs were extracted from tomato seedlings using TRIZol (Invitrogen), and mRNA (1.0 μg) was isolated with an mRNA isolation kit (Promega) and used for cDNA library construction. The tomato cDNA library was screened with BD-βC1 as bait in Saccharomyces cerevisiae strain AH109 (Clontech), and positive clones were selected on a His-deficient medium, confirmed by β-GAL assays.

The plasmids BD-βC1 and AD-SlSnRK1 were cotransformed into S. cerevisiae strain AH109. Plasmids BD-33 and AD-T served a positive controls, and plasmids BD-Lam and AD-T, BD-βC1 and AD, and BD and AD-SlSnRK1 worked as negative controls. Transformants were grown at 30°C for 72 h on synthetic medium lacking Leu and Trp and then transferred to the medium lacking His, Leu, and Trp and containing 5 mM 3-amino-tiazole to identify binding activity. Three independent experiments were performed to confirm the result.

The recombinant plasmids AD-AKIN11 and BD-βC1, SlSnRK1 deletion mutant and AD-βC1, and βC1 deletion mutant and BD-SlSnRK1 were cotransformed into S. cerevisiae Y2HGold cells (Clontech). Transformants were transferred to DDO/Aha to identify binding activity.

BiFC Assay

pC1-YFP1 and pSlSnRK1-YFP2 were introduced individually into Agrobacterium tumefaciens strain C58C1 by electroporation. BiFC experiments were performed as described previously (Yang et al., 2007). YFP fluorescence was observed and photographed by confocal microscopy (Leica TCS S5P) at 48 to 72 h after infiltration.

Subcellular Localization of Proteins

pCh3-GFP and pCh3-GFP-SlSnRK1 were introduced individually into A. tumefaciens strain EHA105 by electroporation. Leaves of 4-week-old N. benthamiana plants were infiltrated with the constructs as described (Liu et al., 2009). About 48 h after infiltration, 1-cm² leaf explants were excised and GFP fluorescence was examined in epidermal cells by confocal microscopy (Leica TCS S5P).
Real-Time RT-PCR Analyses

Total RNA was extracted using TRIzol (Invitrogen). The first-strand cDNA was synthesized as described (Barton et al., 2000; Liu et al., 2002; Tao and Zhou, 2004). Real-time RT-PCR were performed as described (Huang et al., 2009) using primer pair SlnSnRK1-1F/SlnSnRK1-1R specific for SlnSnRK1. The primer SlnSnRK1-5F/SlnSnRK1-5R annealed to the untranslated region of SlnSnRK1 to ensure that only the SlnSnRK1 mRNA gene was amplified. The EF-1a gene was used as an internal control.

Yeast Complementation Assay

All the constructs for yeast complementation assays were transformed into freshly prepared S. cerevisiae strain S14301 (MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 ade2Δ0) competent cells. Yeast complementation experiments were performed (Hao et al., 2003; Shen and Hanley-Bowdoin, 2006).

Protein Expression and Kinase Assay

Recombinant proteins were produced in Escherichia coli strain BL21 (DE3) induced with 0.5 mM isopropyl β-D-thiogalactoside for 16 h at 16°C. Bacterial cells were collected and disrupted by sonication. The GST-fused proteins were purified using GST-binding resin (Novagen, Merck) according to the manufacturer’s instructions.

In vitro kinase assays were performed as described (Lin et al., 2009) with minor modifications. Purified proteins including GST-GRiK and GST-SnRK1-KD were co-conjugated with GST-tagged wild-type βC1 or its mutants (βC1SNRK1, βC1SNRK1C1S33D, βC1SNRK1C1S33A, βC1SNRK1T78A, and βC1SNRK1T78D) in reaction buffer (20 mM Tris-HCl, pH 7.5, 5 mM MgCl2, 10 μM ATP, 0.1 mM CaCl2, and 2 mM dithiothreitol) in a total volume of 20 μL. GST-SAMS and GST were used as positive and negative controls, respectively. Reactions were initiated by the addition of 5 pM of [γ-32P]ATP and transferred to 30°C for 30 min. Loading buffer (6× SDS; 4 μL) was added to terminate the reactions. After boiling at 95°C for 5 min, proteins were separated on a 12% SDS-PAGE gel followed by staining with Coomassie Brilliant Blue R-250. Radioactive signals were visualized through autoradiography and quantified by ImageQuant TL (GE Healthcare).

Virus Inoculation and Infectivity Tests

The infectious clones of TYLCCNB mutants were introduced into A. tumefaciens strain EHA105 by the electroporation method. Wild-type or transgenic N. benthamiana plants expressing Arabidopsis antisense SnRK1 (AS-12) or Arabidopsis sense SnRK1 (S-5) were agroinoculated with an A. tumefaciens carrying TYLCCNV and TYLCCNB mutants.

DNA Gel Blotting

Total DNA was extracted from leaves of tobacco (Nicotiana benthamiana) plants as described previously (Zhou et al., 2003). DNA gel blotting was performed as described previously (Cui et al., 2004). Genomic DNA was stained using ethidium bromide as a loading control.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession number AF143743 (SlnSnRK1).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. SlnSnRK1 has sequence homology with other SnRK1 α-subunits and AMPK from Caenorhabditis elegans.

Supplemental Figure S2. Interaction between AKIN1 and βC1 in yeast.

Supplemental Table S1. Recombinant plasmids used in this study.

Supplemental Table S2. Primers used in plasmid construction and other experiments.

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