Shen et al. Interaction between SlSnRK1 and βC1 protein

1 **RUNNING HEAD:** Interaction between SlSnRK1 and βC1

2 **CORRESPONDING AUTHOR:** Xueping Zhou

3 Institute of Biotechnology, Zhejiang University, Hangzhou 310029,

4 People’s Republic of China

5 Phone: 0086-571-86971680

6 Fax: 0086-571-86971498

7 E-mail: zzhou@zju.edu.cn

8 **RESEARCH CATEGORY:** Plants Interacting with Other Organisms
Tomato SlSnRK1 Protein Interacts with and Phosphorylates βC1, a Pathogenesis Protein Encoded by a Geminivirus Betasatellite

Qingtang Shen, Zhou Liu, Fengming Song, Qi Xie, Linda Hanley-Bowdoin, Xueping Zhou*

State Key Laboratory of Rice Biology, Institute of Biotechnology, Zhejiang University, Hangzhou 310029, People’s Republic of China (Q.S., Z.L., F.S., X.Z.); State Key Laboratory of Plant Genomics, National Center for Plant Gene Research, Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, Beijing 100101, China (Q.X.); Department of Molecular and Structural Biochemistry, North Carolina State University, Raleigh, North Carolina 27695-7622 (L.H.-B.)
FOOTNOTES

1 This work was supported by the National Key Basic Research and Development Program of China (2012CB114004) and the National Science Foundation (Grant No 30530520).

2 These two authors contributed equally to this paper.

* Corresponding author; Email zzhou@zju.edu.cn; Fax 0086-571-86971498.

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (http://www.plantphysiol.org) is: Xueping Zhou (zzhou@zju.edu.cn).
ABSTRACT

The βC1 protein of Tomato yellow leaf curl China betasatellite (TYLCCNB) functions as a pathogenicity determinant. To better understand the molecular basis of βC1 in pathogenicity, a yeast two-hybrid screen of a tomato cDNA library was carried out using βC1 as bait. βC1 interacted with a tomato SNF1-related kinase designated as SlSnRK1. Their interaction was confirmed using a bimolecular fluorescence complementation assay in Nicotiana benthamiana cells. Plants over-expressing SnRK1 were delayed for symptom appearance and contained lower levels of viral and satellite DNA, while plants silenced for SnRK1 expression developed symptoms earlier and accumulated higher levels of viral DNA. In vitro kinase assays showed that βC1 is phosphorylated by SlSnRK1 mainly on serine at position 33 (S-33) and threonine at position 78 (T-78). Plants infected with βC1 mutants containing phosphorylation-mimic aspartate residues in place of S-33 and/or T-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 SlSnRK1 protein attenuates geminivirus infection by interacting with and phosphorylating the βC1 protein.
INTRODUCTION

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 post-transcriptional gene silencing (Whitham et al., 1994; Vanitharani et al., 2005). The elucidation of host-virus interactions will be important for providing additional clues into 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 (Mastrevirus, Topocuvirus, Curtovirus and 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 (Fauquet et al., 2003; Hanley-Bowdoin et al., 2000). In recent years, a betasatellite 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; Briddon et al., 2001; Jose and Usha, 2003; Saunders et al., 2003; Cui et al., 2004). Full-length betasatellite molecules encode an approx. 13.5 kDa protein known as βC1 on the complementary-sense strand. βC1 is a pathogenicity determinant and a suppressor of RNA silencing (Jose and Usha, 2003; Cui et al., 2004; Saunders et al., 2004; Cui et al., 2005; Qian and Zhou, 2005; Saeed et al., 2005; Gopal et al., 2007; Kon et al., 2007). Previous studies showed that βC1 interacts with ASYMMETRIC LEAVES 1 (AS1) to alter leaf development and suppress selected jasmonic acid responses (Yang et al., 2008). βC1 also interacts with a host ubiquitin-conjugating enzyme SlUBC3 and modifies the host ubiquitination system (Eini et al., 2009). Here, we show that the βC1 of Tomato yellow leaf curl China betasatellite (TYLCCNB) interacts with a tomato SUCROSE NONFERMENTING1-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 (TYLCCNB-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 5×10^5 independent, double transformants assayed for histidine 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 according to the scientific name of tomato (Solanum lycopersicum). 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 1545 nucleotides and encodes a protein of 514 amino acids (58,824 daltons). It is closely related to SnRK1 in Nicotiana benthamiana, NPK5 in N. tabacum, and AKIN11 in Arabidopsis thaliana with identities of 95%, 87% and 78%, respectively (Fig. S1 and Fig. 1B). As a typical member of 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 auto-inhibitory sequence (AIS) domain, as well as a C-terminal domain (CTD) that is responsible for β subunit binding and formation of 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 YFPN (pβC1-YFPN) and SlSnRK1 was fused to YFPC (pSlSnRK1-YFP C). Pairwise expression of pβC1-YFP N and pSlSnRK1-YFP C resulted in a YFP fluorescence signal in the cytoplasm of agro-infiltrated cells at 72 h post infiltration, but no YFP fluorescence was observed when pβC1-YFP N and pYFP C, or pSlSnRK1-YFP C and pYFP N were co-expressed (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 localization of that GFP-tagged SlSnRK1 to both the cytoplasmic and nuclear compartments of plant cells.

To determine the expression pattern of SlSnRK1 mRNA, quantitative 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 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 than in TYLCCNV-infected leaves at 3 days 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, A and B), in yeast two-hybrid assays. As shown in Figure 5A, yeast transformants harboring SlSnRK1 mutants M1 (amino acids 281 to 514), M2 (amino acids 1 to 339), M4 (amino acids 1 to 460), M5 (amino acids 1-360 fused with 450-514) or M6 (amino acids 1-290 fused with 339-514) grew on TDO/Aba+ plates, indicative of interaction with βC1. In contrast, mutant M3 (amino acids 1 to 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-terminus regions are involved in binding βC1.

To further define the βC1 binding domain, we constructed 3 mutants (M7 to M9) that divided SlSnRK1-M1 into known functional domains. Yeast two-hybrid assay revealed that both M7 (residues 281 to 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 domain) 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 N- or C-terminus, failed to interact with full-length SISnRK1 (Fig. 5B), indicating that full-length βC1 is required for interaction with SISnRK1.

**Plant SnRK1 affects TYLCCNV/TYLCCNB infection and alters viral DNA accumulation**

To assess the biological significance of SISnRK1–βC1 interactions *in vivo*, we inoculated wild-type or transgenic N. benthamiana plants carrying an Arabidopsis antisense SnRK1 expression cassette (AS-12) or an Arabidopsis 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 over-expressing 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 over-expressing, silenced and wild-type plants (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 over-expressing 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 reduces infection efficiency while lowering SnRK1 levels enhances efficiency. To rule out the possible functional differences between Arabidopsis SnRK1 (AKIN11) and SISnRK1, we showed that βC1 also interacts with AKIN11 (Fig. S2).

**Impacts of SISnRK1 kinase activity by βC1**

We next asked if βC1 can impact SISnRK1 kinase activity in yeast cells. In initial experiments, we tested whether SISnRK1 can complement the yeast snf1 deletion strain △snf1 BY4741 that cannot grow on medium containing a carbon source other than glucose. Expression of SISnRK1 in △snf1 BY4741 restored growth on synthetic complete medium containing 2% (w/v) galactose and 2% (w/v) sucrose as carbon sources, but △snf1 BY4741 transformed with an empty expression plasmid pESC-Ura or a plasmid expressing the kinase-dead mutant CTU-SISnRK1K48R failed to grow on the medium (Fig. 7A). Thus, SISnRK1 can functionally complement SNF1 in yeast and SISnRK1 kinase...
activity is essential for complementation. We found that Δsnf1 BY4741 co-transformed with
SCU-SISnRK1 and SCL-βC1 grew similarly as Δsnf1 BY4741 co-transformed SCU-SISnRK1 and
pESC-Leu (positive control) on the medium containing galactose and sucrose as carbon sources, but Δ
snf1 BY4741 co-transformed with pESC-Ura and SCL-βC1 (negative control) could not grow on the
medium (Fig. 7B), suggesting that βC1 can not inhibit SISnRK1 activity.

SISnRK1 phosphorylates βC1 mainly on threonine at position 78 and serine at position 33 in vitro
To test if SISnRK1 phosphorylates βC1 protein, both proteins were expressed in E. coli as GST
fusions. The SAMS peptide, HMRSAMGLHLVKRR, 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 positive
control, while GST alone was used as a negative control. The Arabidopsis GRIK1 protein has been
reported 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 SISnRK1 during the in vitro kinase
reactions. Because the full-length SISnRK1 protein was insoluble, a truncated SISnRK1 containing the
kinase and UBA domains (SISnRK1-KD) was used for the in vitro phosphorylation assays.
Recombinant βC1, SAMS or GST proteins were separately incubated with GRIK1 and SISnRK1-KD
proteins under the same reaction conditions. Phosphorylation of βC1 was clearly observed after
co-incubation with SISnRK1-KD whereas GST was not phosphorylated (Fig. 8A), indicating that βC1
is a SISnRK1-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 SISnRK1-KD
was carried out. The autoradiography only detected a radiolabeled band for GRIK1, indicating that the
viral protein βC1 is specifically phosphorylated by SISnRK1.

To determine the βC1 residue(s) phosphorylated by SISnRK1 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 threonine at position 78 (T-78)
and serine at position 33 (S-33) are potential phosphorylation sites for serine/threonine kinases (data
not shown). We generated six βC1 point mutants including βC1S33A, βC1S33D, βC1T78A, βC1T78D,
βC1S33A/T78A and βC1S33D/T78D, in which S-33 or T-78 of βC1 were individually or simultaneously
replaced by alanine (A) to eliminate phosphorylation or aspartate (D) 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 of S33A, S33D, T78A, T78D, S33A/T78A and S33D/T78D significantly reduced the level of βC1 phosphorylation relative to the wild-type protein. These differences were not due to different loading amounts of βC1 and its mutants (Fig. 8B). The reductions in radioactive signals for βC1<sup>33A</sup> and βC1<sup>33D</sup> were stronger than for βC1<sup>T78A</sup> and βC1<sup>T78D</sup>. These results indicated that both βC1 S-33 and T-78 are SISnRK1 phosphorylation sites. Replacement of S-33 or T-78 with D did not promote βC1 phosphorylation of the other site, indicating that the two sites are unlikely to be synergistic for phosphorylation. Both double mutants βC1<sup>33A/T78A</sup> and βC1<sup>33D/T78D</sup> retained low but measurable phosphorylation signals (37% to 28% of the wild-type βC1, Fig. 8C), suggesting that other βC1 residues might also be phosphorylated by SISnRK1 *in vitro*.

**Effect of βC1 phosphorylation site mutations on virus infection and viral DNA accumulation**

To assess role of βC1 phosphorylation by SISnRK1, S-33 and/or T-78 mutations were introduced into the βC1 gene of a satellite replicon. Six mutant constructs were generated, including single replacements at S-33 or T-78 to alanine (S33A and T78A) or aspartate (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 aspartate 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 co-inoculated with TYLCCNV and a βC1 phosphomimic mutant (S33D, T78D or S33D/T78D) than that in plants co-inoculated with TYLCCNV and wild-type TYLCCNB (Fig. 9D). In contrast, symptom appearance and severity associated with the TYLCCNB alanine 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 alanine mutants versus the wild-type satellite (Fig. 9D). These data 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 co-opted by geminiviruses to facilitate infection (Kong et al., 2000; Egelkrout et al., 2001; Carvalho et al., 2008a). In contrast, only 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 cells (Xie et al., 1999). Similarly, expression of sense RNAs of tomato SUMO (LeSUMO) impairs Tomato golden mosaic virus (TGMV) replication, suggesting plant SUMO may also play an important role in plant antiviral defense response (Castillo et al., 2004).

TYLCCNV is a monopartite begomovirus associated with betasatellite (TYLCCNB) identified in China (Zhou et al., 2003). We demonstrated previously that TYLCCNV alone produces asymptomatic infections in tobacco, tomato and petunia and that TYLCCNB is required for 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 SISnRK1 by yeast two-hybrid system and BiFC assay.

Yeast SNF1, mammalian AMPK and plant SnRK1 are a group of serine/threonine 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; Halford et al., 2004; Hardie, 2007; Polge and Thomas, 2007; Baena-Gonzalez 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 cell cycle, modulation of development, and response to abiotics or biotics stress (Baena-Gonzalez 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 (BCTV, genus Curtovirus) interact with Arabidopsis SnRK1 (AKIN11) and that AL2 and L2 inactivate SnRK1 leading to enhanced susceptibility. These results suggested that metabolic alterations mediated by

www.plantphysiol.org
SnRK1 may contribute to plant innate antiviral defenses and that SnRK1 inactivation by AL2 and L2 is a counter-defense measure. Although βC1 also binds to SnRK1, we found that βC1 does not inhibit SnRK1 kinase activity and, instead, is phosphorylated by SISnRK1. The βC1 phosphomimic mutants S33D and T78D attenuate symptoms, delay viral infection and reduce viral DNA accumulation, while the alanine 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 (CaMV) (Martinez-Izquierdo and Hohn, 1987) and potyviruses (Ivanov et al., 2001; Fernandez-Fernandez et al., 2002) as well as movement protein (MP) of Tobacco mosaic virus (TMV) (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 the nuclear shuttle protein (NSP) 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 the βC1 by SISnRK1 is a counter-defense response against virus infection by host. We demonstrated previously that transgenic *N. benthamiana*, *N. tabacum* and *Arabidopsis* plants expressing the TYLCCNB βC1 gene are stunted and show leaf cupping and curling. The resulting “symptoms” are much more severe than those associated with TYLCCNV+TYLCCNB 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 TYLCCNV+TYLCCNB infection and increases viral DNA accumulation.

An unanswered question is how phosphorylation of the 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 an 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 (HCV) NS5A (Pietschmann et al., 2001), Turnip yellow mosaic virus (TYMV) 66K protein (Héricourt et al., 2000; Jakubiec et al., 2006), or tobamovirus MP (Kawakami et al., 1999) can affect viral protein stability. In addition, protein phosphorylation often plays a role in ubiquitin-mediated proteolysis, and SCF [one type of multisubunit ubiquitin-protein ligases (E3s)] 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 βC1 protein is degraded by the 26S proteasome (Yang et al., 2008) indicates that SlSnRK1 may interact with and phosphorylate βC1 for degradation by the 26S proteasome leading to attenuation of symptoms and reduction of efficiency of viral infection.

In conclusion, we demonstrated that tomato SlSnRK1 protein interacts with and phosphorylates βC1, a pathogenicity factor encoded by a geminivirus betasatellite. Future studies will determine whether phosphorylation of βC1 negatively impacts its function as an RNA silencing suppressor and/or mediates its degradation by the 26S proteasome.

MATERIALS AND METHODS

Plant Material, Growth Conditions, and Genotyping

*Solanum lycopersicum* CV Hongbaoshi was used to construct the tomato cDNA library. Antisense (As-12) or sense (S-5) AKIN11 transgenic *N. benthamiana* line (Hao et al., 2003) were kindly provided by Dr. David M. Bisaro (Department of Molecular Genetics, the Ohio State University, OH, USA). 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 light/8 h 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 analysis, the coding sequence of the full length βC1
protein and eight deletion fragments containing four N-terminal deletion mutants βC1-M1 (residues 1-118), 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 Table S2, with a tandem repeat construct of TYLCCNB in pBINplus (Cui et al., 2004) as the template. The PCR fragments were inserted into the EcoRI-BamHI sites of the yeast GAL4 binding domain vector pGBKT7 or GAL4 activation domain vector pGADT7 (Clontech, Mountain View, CA, USA), resulting in the recombinant plasmids listed in Table S1, respectively.

The coding sequence of intact SIbSnRK1 was amplified from a tomato leaf cDNA at the six-leaf stage with primer pairs SIbSnRK1-1F-Ec/SIbSnRK1-1R-Ba, and seven deletion mutant fragments of SIbSnRK1 containing N-terminal truncated mutant SIbSnRK1-M1 (residues 281-514), C-terminal truncated mutants SIbSnRK1-M2 (residues 1-339), SIbSnRK1-M3 (residues 1-280) and SIbSnRK1-M4 (residues 1-460), and central mutants SIbSnRK1-M7 (residues 281-339), SIbSnRK1-M8 (residues 340-449) and SIbSnRK1-M9 (residues 450-514), were separately amplified with the primers listed in Table S2. Overlap extension PCR was used to generate the two internal deletion mutants SIbSnRK1-M5 (residues 1-514 with deletion of amino acids 361-449, which removes the potential motif corresponding to the autoinhibitory sequence in mammalian AMPK or yeast Snf1) and SIbSnRK1-M6 (residues 1-514 with deletion of amino acids 291-339, which removes the putative ubiquitin associated domain) (Tao et al., 2002). All of the amplified products were inserted into the EcoRI-BamHI site of the vector pGBKT7 or vector pGADT7 (Clontech), resulting in the recombinant plasmids listed in Table S1.

The coding sequences of the full-length Arabidopsis AKIN11 were amplified separately using the primer pairs listed in Table S2. The AKIN11 PCR fragments were digested with EcoRI/BamHI and cloned into the vector pGBKT7 and vector pGADT7 (Clontech) to generate recombinant plasmids listed in Table S1.

For production of bimolecular fluorescence complementation (BiFC) vectors, the full-length coding sequence of βC1 was amplified using the primers listed in Table S2 and cloned into the PacI-AscI site of p2YN (Yang et al., 2007) as a fusion with the N-terminal fragment of YFP resulting in pβC1-YFPN. The full-length coding sequence of tomato SIbSnRK1 was amplified using the primers listed in Table S2 and cloned into the PacI-AscI site of p2YC (Yang et al., 2007) as a fusion with the C-terminal fragment of YFP resulting in pSIbSnRK1-YFPC.
For subcellular localization studies, SlSnRK1 was tagged at its N-terminus with GFP by inserting PCR-amplified SlSnRK1 cDNAs using primer pair listed in Table S2 into the BamHI-PstI site of pCHF3-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 Table S2, and cloned into the EcoRI-SpeI site of pESC-Ura (2μ origin, URA3 selection marker) (Stratagene, La Jolla, CA, USA), or KpnI-BamHI site of pYC2/CT-Ura (CEN6/ARSH4 origin, URA3 selection marker) (Invitrogen, Carlsbad, CA, USA), respectively, resulting in SCU-SlSnRK1 or CTU- SlSnRK1. The dead kinase mutant SlSnRK1K48R was generated by the overlapping PCR (Tao et al., 2002) using complementary primer pairs listed in Table S2. The PCR fragments were digested with KpnI-BamHI and cloned into the vector pYC2/CT-Ura (Invitrogen) to generate recombinant plasmid CTU-SlSnRK1K48R. The coding sequences of intact βC1 were amplified using the primers listed in Table S2 and cloned into the BamHI-SalI site of pESC-Leu (2μ origin, LEU2 selection marker) (Stratagene), resulting in SCL-βC1.

For construction of infectious clones of TYLCCNB-βC1 mutants, site-directed mutagenesis was performed to alter βC1 S-33 coding triplets TCA to alanine (A) coding triplets GCA or aspartic acid (D) coding triplet GAC, and βC1 T-78 coding triplets ACA to alanine (A) coding triplets GCA or aspartic acid (D) coding triplet GAC, or both Ser 33 and Thr 78 to alanine (A) or aspartic acid (D), resulting in mutants βC1S33A, βC1S33D, βC1T78A, 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 complementary primer pairs listed in Table S2. The overlapping PCR products were inserted into the pGEM-T-Easy (Promega, Madison, WI, USA) vector to produce clones pGEMβS33A, pGEMβS33D, pGEMβT78A, and pGEMβT78D, respectively. The double mutations pGEMβS33A/T78A or pGEMβS33D/T78D was generated by overlapping PCR (Tao et al., 2002) with complementary primer pairs Y10βC1S33A-F/Y10βC1S33A-R or Y10βC1S33D-F/Y10βC1S33D-R using pGEMβT78A or pGEMβT78D 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 construction of infectious clones (Table S1) of βC1 mutants.

Plasmids pNSB1554 harboring GST-fused GRIK1 was constructed previously (Shen et al., 2009).
The plasmid pGEX-KG-SAMS expressing a positive control peptide SAMS for kinase assay was kindly provided by Dr. David M. Bisaro (Department of Molecular Genetics, the Ohio State University, OH, USA).

To generate the GST-SlSnRK1-KD expression vector, the plasmid pGADT7-SlSnRK1-M2 was digested with EcoRI and XhoI and then inserted into pGEX-4T-1 vector (GE healthcare, Piscataway, NJ, USA). To construct the GST-tagged TYLCCNB-βC1 for in vitro kinase assay, pGEMβ, pGEMβC1S33A, pGEMβC1S33D, pGEMβC1T78A, pGEMβC1T78D, pGEMβC1S33A/T78A, or pGEMβC1S33D/T78D was used as PCR template with primer pairs Y10betaC1-1F-Ec/Y10betaC1-1R-Xh. The amplified fragments were inserted into the EcoRI-XhoI 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 Manuals (Clontech). Total RNAs were extracted from tomato seedlings using TRIzol (Invitrogen), and mRNA (1.0 mg) 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 histidine-deficient medium, confirmed by β-gal assays.

The plasmids BD-βC1 and AD-SISnRK1 were co-transformed into *S. cerevisiae* strain AH109. Plasmids BD-53 and AD-T were served a positive controls, and BD-Lam and AD-T, BD-βC1 and AD, and BD and AD-SISnRK1 were used 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-aminotriazole (3-AT) to identify binding activity. Three independent experiments were performed to confirm the result.

The recombinant plasmids AD-AKIN11 and BD-βC1, SISnRK1 deletion mutant and AD-βC1, or βC1 deletion mutant and BD-SISnRK1 were co-transformed into *S. cerevisiae* Y2HGold cells (Clontech). Transformants were transferred to TDO/AbA (SD/-His/-Leu/-Trp media in the present of 90 or 120 ng/mL Aureobasidin A) to identify binding activity.

**BiFC Assay**

pβC1-YFPN and pSISnRK1-YFPC 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 (CLSM, Leica TCS SP5, Mannheim, Germany) at 48–72 h after infiltration.

**Subcellular Localization of Proteins**

pCHF3-GFP and pCHF3-GFP-SlSnRK1 were introduced individually into *A. tumefaciens* strain EHA105 by electroporation. Leaves of 4-weeks-old *N. benthamiana* plants were infiltrated with the *A. tumefaciens* harboring 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 (CLSM, Leica TCS SP5, Mannheim, Germany).

**Real-time RT-PCR Analyses**

Total RNA was extracted using TRIzol (Invitrogen). The first-strand cDNA was synthesized as described (Burton 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 SlSnRK1-rt-ORF-1F/SlSnRK1-rt-UTR-1r specific for SlSnRK1. The primer SlSnRK1-rt-UTR-1r annealed to the untranslated region of SlSnRK1 gene to ensure that only the SlSnRK1 mRNA gene was amplified. The EF-1-α gene was used as an internal control.

**Yeast Complementation Assay**

All the constructs for yeast complementation assays were transformed into freshly prepared *S. cerevisiae snf1* deletion strains BY4741 (MATα his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 snf1Δ) (Biosystems, Huntsville, AL, USA) competent cells. Yeast complementation experiments were performed as described (Hao et al., 2003; Shen and Hanley-Bowdoin, 2006).

**Protein Expression and Kinase Assay**

Recombinant proteins were produced in *E. 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 KGaA, Darmstadt, Germany) 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-SlSnRK1-KD, were co-incubated with GST tagged
wild-type βC1 or its mutants (βC1S33A, βC1S33D, βC1T78A, βC1T78D, βC1S33A/T78A, βC1S33D/T78D) in reaction buffer (20 mM Tris-HCl, pH 7.5, 5 mM MgCl₂, 10 μM ATP, 0.1 mM CaCl₂, 2 mM DTT) in a total volume of 20 μL. GST-SAMS and GST were used to serve as positive and negative controls, respectively. Reactions were initiated by addition of 5 μCi γ-32P-ATP and transferred to 30°C for 30 min. 6×SDS loading buffer (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 and followed by staining with Coomassie Brilliant Blue R-250. Radioactive signals were visualized through autoradiography and quantified by ImageQuant TL V2003 software (GE Healthcare).

Virus Inoculation and Infectivity Tests

The infectious clones of TYLCCNB mutants were introduced into A. tumefaciens strain EHA105 by electroporation method. The wild-type or transgenic N. benthamiana plants expressing Arabidopsis antisense SnRK1 (AS-12) or Arabidopsis SnRK1 (S-5) were agroinoculated with an overnight culture of A. tumefaciens carrying TYLCCNV and TYLCCNB (pBinPLUS-1.7A and pBinPLUS-2β) (Cui et al., 2004) or TYLCCNB mutants. The course of infection was monitored as described (Carvalho et al., 2008b). DPI 50% was determined using data from three independent experiments.

DNA Gel Blotting

Total DNA was extracted from leaves of tobacco plants as previously described (Zhou et al., 2001). DNA gel blotting was performed as described previously (Cui et al., 2004). Genomic DNA was stained using ethidium bromide as a loading control.

Supplemental Data

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

Supplemental Figure S1. SISnRK1 has sequence homology with other SnRK1-α subunits and AMPK from Caenorhabditis elegans.

Supplemental Figure S2. Interaction between AKIN11 and βC1 in yeast.

Supplemental Table S1. Recombinant plasmids used in this study.

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

ACKNOWLEDGMENTS

We thank Dr. David M. Bisaro, Department of Molecular Genetics, the Ohio State University, for
providing transgenic *Nicotiana benthamiana* line S-5 expressing AKIN11, plasmids p2YN, p2YC and pGEX-KG-SAMS. We also thank Dr Yan Guo, National Institute of Biological Sciences in Beijing for technical help of phosphoration tests.

**LITERATURE CITED**


cell-to-cell movement protein by a developmentally regulated plant cell wall-associated protein kinase. Genes Dev 7: 904-910


Fauquet CM, Stanley J (2005) Revising the way we conceive and name viruses below the species level: a review of geminivirus taxonomy calls for new standardized isolate descriptors. Arch Virol 150: 2151-2179


Interaction between SISnRK1 and βC1 protein


6 Halford NG, Hey SJ (2009) Snf1-related protein kinases (SnRKs) act within an intricate network that links metabolic and stress signalling in plants. Biochem J 419: 247-259


Interaction between SI SnRK1 and βC1 protein

Shen et al.

1. geminivirus DNA1 component. Plant Biotechnol J 7: 254-265


Shen et al. Interaction between SiSnRK1 and βC1 protein

1. binding to coat protein of Broad bean wilt virus 2 VP37 protein. Virus Res 143: 86-93
Shen et al. Interaction between SISnRK1 and βC1 protein

1 Virology 324: 37-47


Shen et al.  Interaction between SISnRK1 and βC1 protein


4. Won KA, Reed SI (1996) Activation of cyclin E/CDK2 is coupled to site-specific autophosphorylation and ubiquitin-dependent degradation of cyclin E. EMBO J 15: 4182-4193


Figure legends

Figure 1. A. Interaction between SISnRK1 and TYLCCNB-βC1 in a yeast two-hybrid system. Yeast strain AH109 co-transformed with the indicated plasmids was spotted with 10-fold serial dilutions on SD/-His/-Leu/-Trp medium containing 5 mM 3-aminotriazole (3-AT). B. Phylogenetic tree based on SISnRK1 amino acid sequences shown in Figure S1 using Clustal analysis with PAM250 residue weight (DNASTAR Inc., Madison, WI, USA). C. Schematic representation of SISnRK1. Putative functional domains are indicated. The KD (kinase domain), UBA (ubiquitin associated domain), AIS (auto-inhibitory sequence) and CTD (C-terminal domain responsible for β subunit binding and formation of SnRK1 complex) were deduced from InterProScan online software (http://www.ebi.ac.uk/Tools/InterProScan/) and further confirmed by comparison with previous descriptions (Crute et al., 1998; Hardie, 2007).

Figure 2. BiFC visualization of interaction between TYLCCNB-βC1 and SISnRK1 in N. benthamiana leaves. a, YFP fluorescence; b, bright field; c, YFP/bright field overlay. Scale bars represent 50 μm.

Figure 3. Subcellular localization of SISnRK1 in N. benthamiana epidermal cells. Micrographs showing cells expressing GFP (upper panel) or GFP:SISnRK1 (bottom panel) were examined under fluorescent-field (left), bright-field (middle), or an overlay of bright and fluorescent illumination (right) by a confocal microscope. Arrows indicate nuclei. Scale bars are 50 μm.

Figure 4. SISnRK1 mRNA levels in various tomato tissues (A) or TYLCCNV/TYLCCNB and TYLCCNV infected plants at 3 days post inoculation (B). Relative mRNA levels in tomato tissues were normalized using EF-1-α mRNA as reference. Values are means of three independent experiments. Different lowercase letters on the bars denote significant differences (Fisher’s LSD method, P<0.05).

Figure 5. Identification of the binding domains responsible for SISnRK1-TYLCCNB-βC1 interaction. A. Schematic representation of the truncated mutants of SISnRK1 and the yeast two-hybrid analysis of their interactions with βC1. The yellow box represents the kinase domain (KD), the gray box represents the UBA domain, the light blue box represents the AIS and the red box represents the CTD domain. B. Diagram of deletion mutants of βC1 used to determine the binding requirements for SISnRK1. The serine residue at position 33 is indicated as a dark blue box and the threonine residue at position 78 is labeled as a green box.

Figure 6. TYLCCNV/TYLCCNB infectivity efficiency and viral DNA accumulation levels in wild-type (WT) and transgenic plants expressing antisense SnRK1 (AS-12) or sense SnRK1 (S-5).
Shen et al.  

Interaction between SISnRK1 and βC1 protein

Course of infection in AS-12, S-5 and WT lines. Values represent the percentages of systemically infected plants at different days postinoculation (DPI). B. Days post-inoculation to reach 50% of infected plants (DPI 50%) in infected AS-12, S-5 and wide type plants. Different lowercase letters on the bars denote significant differences (Fisher’s LSD method, P<0.05). All data represent means ± SD of triplcate experiments. In each experiment, 12 plants of each line were inoculated with the A. tumefaciens strain EHA105 culture containing TYLCCNV and TYLCCNB. C. TYLCCNV and TYLCCNB DNA levels in wild-type (WT) 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.

Figure 7. SISnRK1 functionally complements SNF1 in yeast (A) and TYLCCNB-βC1 does not inhibit SISnRK1 activity in yeast (B). Cells of △snf1 BY4741 transformed with the indicated plasmids were spotted with serial 10-fold dilutions on selective synthetic complete medium. SISnRK1 was expressed from a high-copy plasmid (SCU-SISnRK1) or a low-copy plasmid (CTU-SISnRK1). βC1 was expressed from a high-copy plasmid (SCL-βC1) and SISnRK1K48R was expressed from a low-copy plasmid (CTU-SISnRK1K48R). △snf1 BY4741 transformed with an empty vector pESC-Ura or co-transformed with pESC-Ura and SCL-βC1 were used as negative controls. △snf1 BY4741 co-transformed with SCU-SISnRK1 and pESC-Leu served as a positive control.

Figure 8. In vitro phosphorylation of βC1. A. SISnRK1 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 weights, GST-SAMS (approximately 27 kDa) co-migrates with GST (about 26 kDa) during electrophoresis. B. SISnRK1 phosphorylates βC1 primarily at S-33 and T-78. 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 on the bars denote significant differences (Fisher’s LSD method, P<0.05).

Figure 9. Effects of βC1 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, TYLCCNB-S33D/T78D) at 17 days postinoculation (DPI). CK- is the mock-inoculated plant. B. Infection course of TYCCNV and TYCCNB or its mutants. Values represent the 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. Days post-inoculation to reach 50% of infected plants (DPI 50%) after inoculation of TYLCCNV and TYLCCNB or its mutants. The data are means ± SD of triplicate experiments. Different lowercase letters on the bars denote significant differences (Fisher’s LSD method, P<0.05). D. TYLCCNV and TYLCCNB DNA levels in wild-type (WT) 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.
A

AD-SISnRK1 + BD-βC1
AD + BD-βC1
AD-SISnRK1 + BD
AD-T + BD-Lam
AD-T + BD-53

SD/-His/-Leu/-Trp + 5 mM 3-AT

fold dilution

10^-1  10^-2  10^-3  10^-4

B

Tomato SISnRK1
Nicotiana benthamiana SnRK1
Nicotiana attenuata SnRK1
Nicotiana tabacum NPK5
Arabidopsis AKIN10
Arabidopsis AKIN11
Oryza sativa SnRK1
Tomato SNF1-related protein kinase
Caenorhabditis elegans AMPK

46.4

C

SISnRK1

KD

UBA

AIS

α-CTD