

A NAC Domain Protein Interacts with *Tomato leaf curl virus* Replication Accessory Protein and Enhances Viral Replication

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Geminivirus replication enhancer (REn) proteins dramatically increase the accumulation of viral DNA species by an unknown mechanism. In this study, we present evidence implicating SINAC1, a new member of the NAC domain protein family from tomato (*Solanum lycopersicum*), in *Tomato leaf curl virus* (TLCV) REn function. We isolated SINAC1 using yeast (*Saccharomyces cerevisiae*) two-hybrid technology and TLCV REn as bait, and confirmed the interaction between these proteins in vitro. TLCV induces SINAC1 expression specifically in infected cells, and this upregulation requires REn. In a transient TLCV replication system, overexpression of SINAC1 resulted in a substantial increase in viral DNA accumulation. SINAC1 colocalized with REn to the nucleus and activated transcription of a reporter gene in yeast, suggesting that in healthy cells it functions as a transcription factor. Together, these results imply that SINAC1 plays an important role in the process by which REn enhances TLCV replication.

INTRODUCTION

Geminiviruses are a large and diverse family of plant-infecting pathogens segregated into four genera based on genome structure, insect vectors, and host range (van Regenmortel et al., 2000). They possess small, single-stranded DNA genomes comprising one or two components of 2.6 to 2.8 kb. Of the proteins they encode, only the replication-associated protein (Rep) is essential for virus replication. Rep, the product of the *C1* (also designated *AC1*, *L1*, or *AL1*) gene, specifically recognizes and binds the viral origin (Fontes et al., 1994; Behjatnia et al., 1998), and nicks and religates viral DNA to initiate and terminate rolling-circle replication (Laufs et al., 1995). However, it does not have a DNA polymerase function. Therefore, geminiviruses are heavily dependent on host factors to amplify their genome, but many infect differentiated cells that have exited the cell division cycle and cannot support DNA replication (Nagar et al., 1995; Lucy et al., 1996; Sudarshana et al., 1998; Morra and Petty, 2000). As a consequence, an early step in the geminivirus infection process is induction of the required replication machinery.

Reprogramming of the plant cell to facilitate geminivirus replication appears to rely heavily on an interaction between Rep and retinoblastoma (Rb; Xie et al., 1995; Collin et al., 1996; Grafi et al., 1996; Ach et al., 1997). In animal cells, Rb modulates

the activity of E2F transcription factors, which are involved in the transcriptional regulation of genes expressed at the G1/S boundary of the cell division cycle (Harbour and Dean, 2000). Mammalian DNA oncoviruses encode proteins that interact with Rb and consequently disrupt the Rb control pathway (Chellappan et al., 1992; Zamanian and La Thangue, 1992). The idea that geminiviral Rep proteins may act in an analogous manner was first suggested from the observation that induction of *proliferating cell nuclear antigen* (*PCNA*) occurred in transgenic plants expressing Rep from *Tomato golden mosaic virus* (TGMV; Nagar et al., 1995). *PCNA* is an accessory factor for DNA polymerase δ that is normally present only in S-phase cells (Daidoji et al., 1992). Two lines of evidence imply that induction of *PCNA* is mediated by the Rep–Rb interaction. First, analysis of Rep mutants revealed that the ability of Rep to activate *PCNA* expression is linked tightly to its capacity to interact with Rb (Kong et al., 2000). Second, induction of *PCNA* occurs at the transcriptional level, and the *PCNA* promoter is under E2F negative control (Egelkrout et al., 2001).

It is becoming increasingly evident that other host factors are involved in geminivirus replication. Xie et al. (1999) identified two wheat (*Triticum aestivum*) proteins, *GRAB1* and *GRAB2*, which interact with *Wheat dwarf virus* (WDV) RepA. Overexpression of these proteins in cultured cells inhibited WDV DNA replication, suggesting that RepA disrupts a GRAB-mediated response that represses viral infection. *GRAB1* and *GRAB2* are both members of the recently identified NAC family of genes found in many plant species but, so far, not in other eukaryotes. NAC proteins share a common structure consisting of a conserved N-terminal region (the NAC domain) and a highly variable C terminus. The name is derived from the three type members, *NO APICAL MERISTEM* (*NAM*) from petunia (*Petunia hybrida*; Souer et al., 1996) and the *ATAF* and *CUP-SHAPED COTYLEDON* (*CUC*) genes from

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Article, publication date, and citation information can be found at www.plantcell.org/cgi/doi/10.1105/tpc.104.027235.

Arabidopsis thaliana (Aida et al., 1997). Since the identification of these genes, many more NACs have been found; Ooka et al. (2003) studied the rice (*Oryza sativa*) and *Arabidopsis* genomes and found 75 and 105 predicted NAC proteins in each species, respectively. NACs possess roles as diverse as pattern formation in embryos (Souer et al., 1996), flower development (Sablowski and Meyerowitz, 1998), leaf senescence (John et al., 1997), and auxin-dependent lateral foot formation (Xie et al., 2000). In addition to these developmental roles, they have also been implicated in plant defense responses (Collinge and Boller, 2001).

Geminiviral replication enhancer (REn) proteins (also designated C3, AC3, L3, or AL3) are able to increase viral DNA accumulation (Elmer et al., 1988; Sunter et al., 1990) and enhance infectivity and symptom expression (Hormuzdi and Bisaro, 1995). Although little molecular information regarding this process is available, replication accessory factors encoded by mammalian oncoviruses often interact with host proteins to generate a cellular environment suited to DNA replication (Jansen-Durr, 1996). Consistent with this idea, REn was recently shown to bind Rb (Settlage et al., 2001), implying that, like Rep, this protein is involved in disruption of cell cycle controls. In this study, we screened a tomato (*Solanum lycopersicum*) library for proteins that interact with the REn protein from the Australian *Tomato leaf curl virus* (TLCV) to determine whether other host factors are involved in REn function. A new member of the NAC domain family, which we have named SINAC1 (for *S. lycopersicum* NAC1), was shown to interact with REn in yeast (*Saccharomyces cerevisiae*) and in vitro. Here, we present evidence implicating SINAC1 in REn-mediated enhancement of viral DNA accumulation.

RESULTS

Identification of a NAC Domain Protein That Interacts with REn

To identify host proteins interacting with the TLCV REn protein, a yeast two-hybrid screen of a tomato cDNA library fused to the B42 activation domain (AD)-encoding sequence (Zhou et al., 1995) was performed using REn fused to the LexA DNA-binding domain (BD) as bait. A total of 2×10^6 transformants were assayed for Leu prototrophic growth and green fluorescent protein (GFP) activity. Of these transformants, one was able to activate both reporter genes. Plasmid DNA was rescued, verified by retransformation into yeast with the bait, and the sequence of the insert determined. The plasmid contained a cDNA of 1304 bp encoding a predicted full-length translation product of 301 amino acids. Nucleotides 52 to 597 of the cDNA are identical to an EST generated from tomato carpel tissue (GenBank accession number AI486942). A BLAST query of the protein sequence revealed that the N-terminal 169 amino acid residues contain the five conserved blocks of homology that comprise the NAC domain (Figure 1A, boxed). Based on this defining characteristic, we named the protein SINAC1 for *S. lycopersicum* NAC1 (GenBank accession number AY498713).

A recent phylogenetic analysis of the NAC domains from known NAC family proteins and putative *Arabidopsis* and rice

NACs separated them into 18 subgroups (Ooka et al., 2003). We compared the NAC domains from SINAC1 and other known NAC family proteins. According to dendograms obtained by the neighbor-joining method (Figure 1B) and the maximum-parsimony method (data not shown), SINAC1 falls into the so-called ATAF subgroup. The C-terminal region of NAC proteins, termed the transcriptional activation region (TAR), is highly divergent, but Ooka et al. (2003) found 13 common motifs (CMs) in 12 of the 18 subgroups. Members of the ATAF subgroup contain the sequence EVQS[E/x]PK[W/I], which is also present in SINAC1 (Figure 1A, boxed and labeled TAR-CM). This supports our classification of SINAC1 into this subgroup. Analysis of the primary sequence of SINAC1 using PSORT II (<http://bioweb.pasteur.fr/seqanal/interfaces/psort2.html>) identified a putative classical (SV40 large T antigen-type) nuclear localization signal in subdomain C from amino acids 74 to 80 (Figure 1A, underlined). This sequence, PRDRKYP, was conserved amongst 12 NACs in a study performed by Kikuchi et al. (2000), suggesting that it may be functional in vivo.

To map the domains responsible for the interaction between REn and SINAC1, truncations of the genes encoding both proteins were made and cloned into pLexA and pB42AD to create fusions with the LexA DNA BD and B42 AD, respectively. The secondary structure of REn, predicted using PSIPRED (<http://bioinf.cs.ucl.ac.uk/psipred/>), contains three α -helices found between amino acids 56 to 65, 79 to 95, and 101 to 116. Because α -helices are frequently important in protein-protein interactions, we generated three truncations of REn based on the location of these putative structures (Figure 2A). REn 1-70 contains only the first helix, REn 40-120 contains all three helices, whereas REn 90-134 contains the third helix and a part of the second. Three truncations of SINAC1 were made based on the location of NAC subdomains (Figure 2A): SINAC1 1-70 contains subdomains A, B, and a small part of C; SINAC1 1-170 contains all of the five subdomains that make up the NAC domain; and SINAC1 71-301 contains subdomains D and E and the majority of C, and all of the variable C terminus.

Each of the REn and SINAC1 truncations as well as the full-length proteins were coexpressed in yeast and their interaction assayed by Leu prototrophic growth and GFP expression. REn 1-70 was able to interact with full-length SINAC1 (Figure 2C), whereas the other two REn truncations could not. This suggests that the first putative α -helix of REn may be involved in SINAC1 binding. None of the three truncations of SINAC1 were able to interact with REn in yeast. This may indicate the involvement of a larger proportion of SINAC1 in the interaction or reflect structural constraints imposed on the functional REn-interacting domain. Immunoblot analysis of yeast cells demonstrated that noninteracting REn and SINAC1 truncations were expressed at levels similar to those of interacting proteins (Figure 2B), confirming that negative results were not as a result of an absence of protein.

We examined the general significance of SINAC1 binding to TLCV REn by testing whether SINAC1 could also interact with REn encoded by TGMV. TGMV is a bipartite begomovirus encoding a REn protein that is 54.2% identical in sequence (65.6% similar) to TLCV REn. In the same yeast two-hybrid assay, TGMV REn also interacted with SINAC1, as shown in

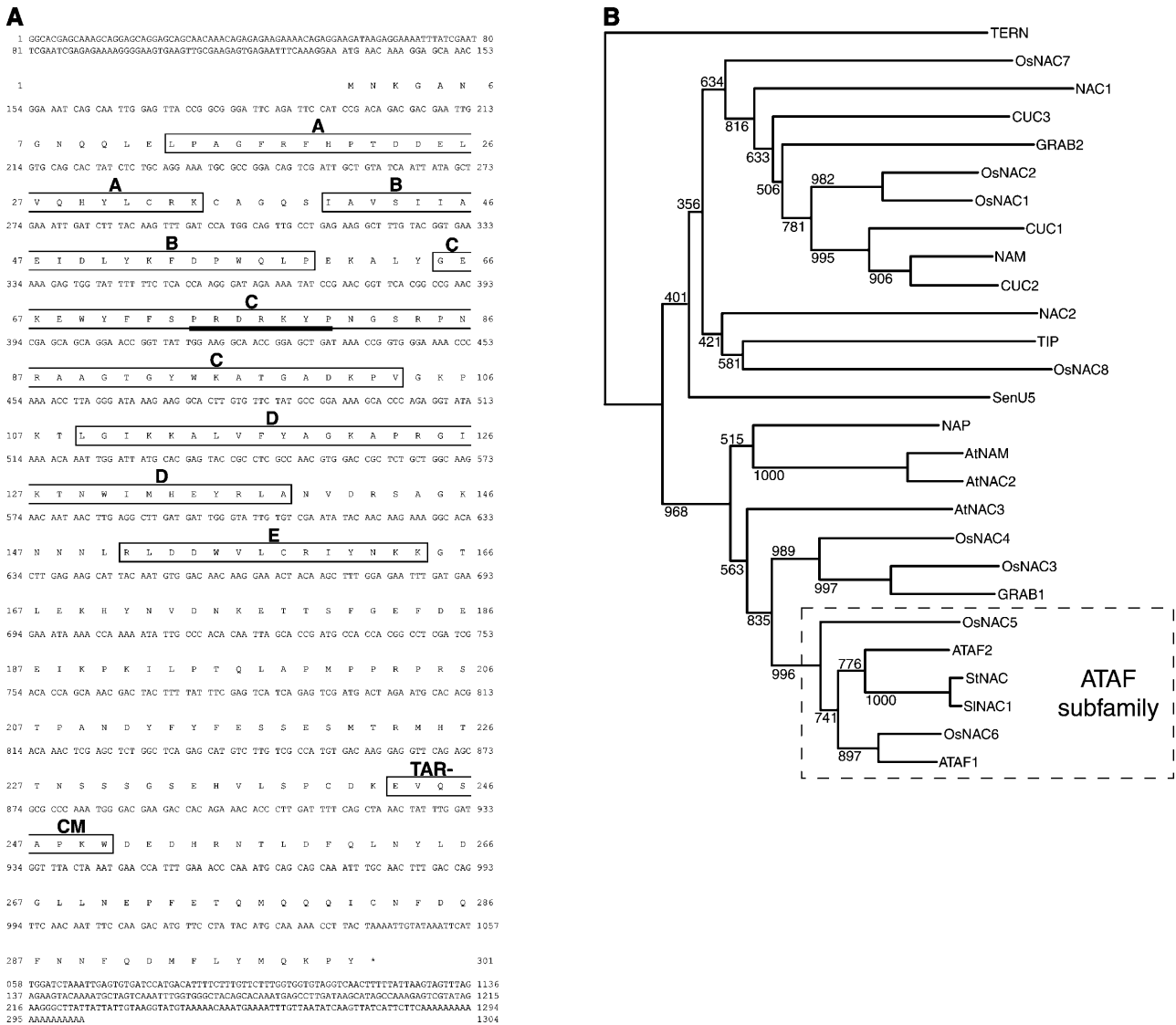


Figure 1. Nucleotide Sequence of *SINAC1* and Alignment of Its Putative Translation Product with Other NAC Domain Proteins.

(A) Nucleotide and amino acid sequences of *SINAC1*. The five subdomains (A to E) comprising the NAC domain are shown in empty boxes. A putative nuclear localization signal is indicated by a bold line under the sequence PRDRKYP. The TAR-CM of the ATAF subgroup is also boxed.

(B) The predicted amino acid sequence of *SINAC1* (Figure 1A) and known NAC family proteins were subjected to phylogenetic analysis. Multiple sequence alignment of the proteins was conducted using ClustalX (Thompson et al., 1997), and phylogenetic analysis was performed by the neighbor-joining method (Saitou and Nei, 1987). A bootstrap analysis of 1000 resampling replicates was conducted with ClustalX. The rooted phylogenetic tree was displayed using the NJPlot program included with ClustalX. The gene names and references for other NACs are as follows: *A. thaliana*, *ATAF1* and *ATAF2* (Aida et al., 1997), *AtNAC2* (Takada et al., 2001), *AtNAC3* (Takada et al., 2001), *AtNAM* (Duval et al., 2002), *CUC1* (Takada et al., 2001), *CUC2* (Takada et al., 2001), *CUC3* (Vroemen et al., 2003), *NAC1* (Xie et al., 2000), *NAC2*, *NAP* (Sablowski and Meyerowitz, 1998), and *TIP* (Ren et al., 2000); rice, *OsNAC1* to *OsNAC8* (Kikuchi et al., 2000); petunia, *NAM* (Souer et al., 1996); tomato, *SenU5* (John et al., 1997); potato, *StNAC* (Collinge and Boller, 2001); and wheat, *GRAB1* and *GRAB2* (Xie et al., 1999).

Figure 3. Neither REn protein interacted with the control protein TLCV C2 (also designated AC2, L2, AL2, or TrAP) nor with the AD alone. A test of *Tomato yellow leaf curl Sardinia virus* (TYLCSV) REn-SINAC1 interaction was not possible because TYLCSV REn exhibited weak autoactivation activity in our yeast system (data not shown).

SINAC1 Acts as a Transcriptional Activator in Yeast

There is considerable evidence to suggest that NAC domain proteins function as transcription factors. First, several NACs, including *ATAF1* and *ATAF2* (Souer et al., 1996), *AtNAM* (Duval et al., 2002), *NAC1* (Xie et al., 2000), *TIP* (Ren et al., 2000), and a group of *Brassica napus* NACs (Hegedus et al., 2003), are able

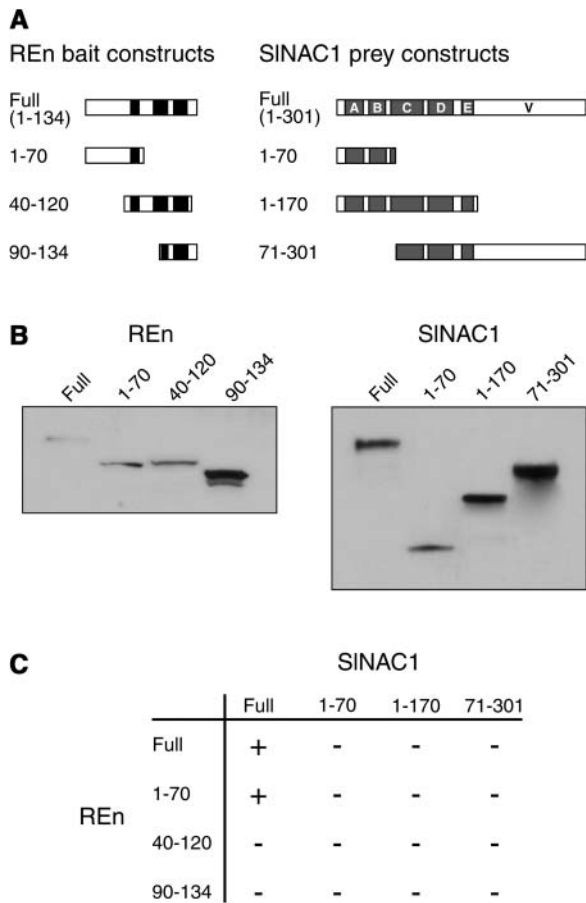


Figure 2. Deletion Analysis of REn and SINAC1 to Identify Regions Required for Interaction between the Two Proteins.

(A) Diagrammatic representation of REn (bait) and SINAC1 proteins (prey) tested for interaction. The REn proteins were expressed as LexA DNA BD fusions, and the SINAC1 proteins were expressed as B42 AD fusions. The positions of three putative α -helices in REn are indicated by closed boxes. In SINAC1, the positions of the NAC subdomains are shown in shaded boxes (A to E), whereas the variable C terminus is denoted V.

(B) Immunoblot analysis of yeast cells demonstrating that noninteracting REn-LexA fusions and SINAC1-B42 fusions are expressed at levels similar to those of interacting fusion proteins. Total protein from yeast cultures containing different REn and SINAC1 fusion proteins was extracted, fractionated on 4 to 20% SDS-polyacrylamide gels, and immunoblotted with anti-LexA (to detect REn-LexA fusions) or anti-hemagglutinin (HA) (to detect SINAC1-B42 fusions).

(C) The N-terminal region of REn is important for SINAC1 binding. Interaction was indicated by the ability of cells transformed with bait, prey, and displayREPORTER plasmids to grow on medium lacking Leu. As an additional indicator of interaction, colonies were monitored for GFP expression by visualization under UV light.

to activate transcription of a reporter gene in yeast, an activity mediated by the divergent C-terminal sequences. Second, AtNAM and NAC1 bind a specific DNA sequence found in the 35S promoter of *Cauliflower mosaic virus* (CaMV; Xie et al., 2000; Duval et al., 2002). Third, overexpression of NAC1 in Arabidopsis caused upregulation of the auxin-responsive genes AIR3 and

DBP (Xie et al., 2000), whereas CUC1 activated the expression of genes involved in the development of the shoot apical meristem (Hibara et al., 2003). Finally, a nuclear localization pattern has been observed for NAC1 (Xie et al., 2000).

Based on these data, we tested for the presence of an AD in SINAC1 using yeast as an assay system. A SINAC1 fusion to the LexA DNA BD was coexpressed with pSH18-34, which contains eight LexA operators that direct transcription of the *lacZ* gene (Golemis et al., 1994). Cells were assayed for β -galactosidase activity using a liquid culture assay (see Methods). As predicted, the LexA:SINAC1 fusion was able to activate expression of the reporter gene, and its transactivation activity was at least as strong as the positive control, a LexA fusion to the GAL4 AD (Figure 4A). Four truncations of the SINAC1 gene were fused to LexA to determine the domains required for transcriptional activation (Figure 4A). This deletion analysis revealed that the variable C-terminal region (amino acids 71 to 301) could activate transcription of *lacZ* but more weakly than full-length SINAC1. None of the N-terminal fragments (1-70, 1-170, and 1-240) were able to promote expression of *lacZ*. These data indicate that SINAC1 has a transcriptional AD that is active in yeast and is

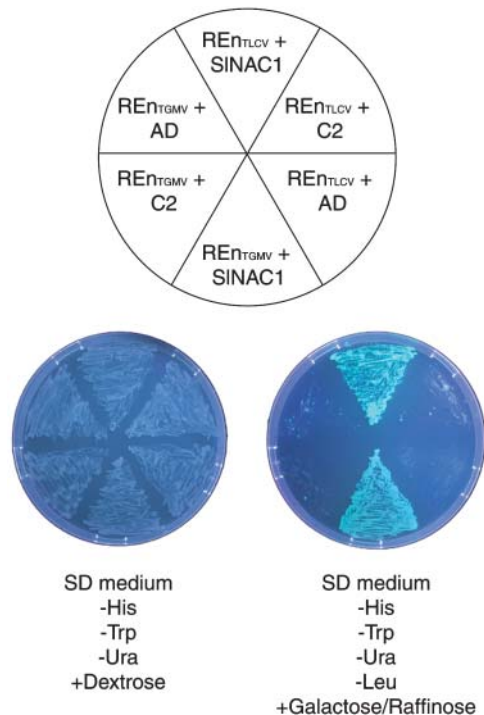


Figure 3. SINAC1 Interacts with Both TLCV and TGMV REn.

Yeast two-hybrid assays testing the ability of SINAC1 to interact with REn of TLCV (REn_{TLCV}) and TGMV (REn_{TGMV}). Yeast coexpressing proteins as indicated (top) were grown on SD – His – Trp – uracil (Ura) medium (bottom left), and interaction was tested by Leu prototrophy and GFP expression on an inductive carbon source (galactose and raffinose; bottom right). REn proteins were fused to the LexA DNA BD, whereas SINAC1 was fused to the B42 AD. Negative controls included REn_{TLCV} and REn_{TGMV} coexpressed with TLCV C2 fused to the AD, or coexpressed with AD alone.

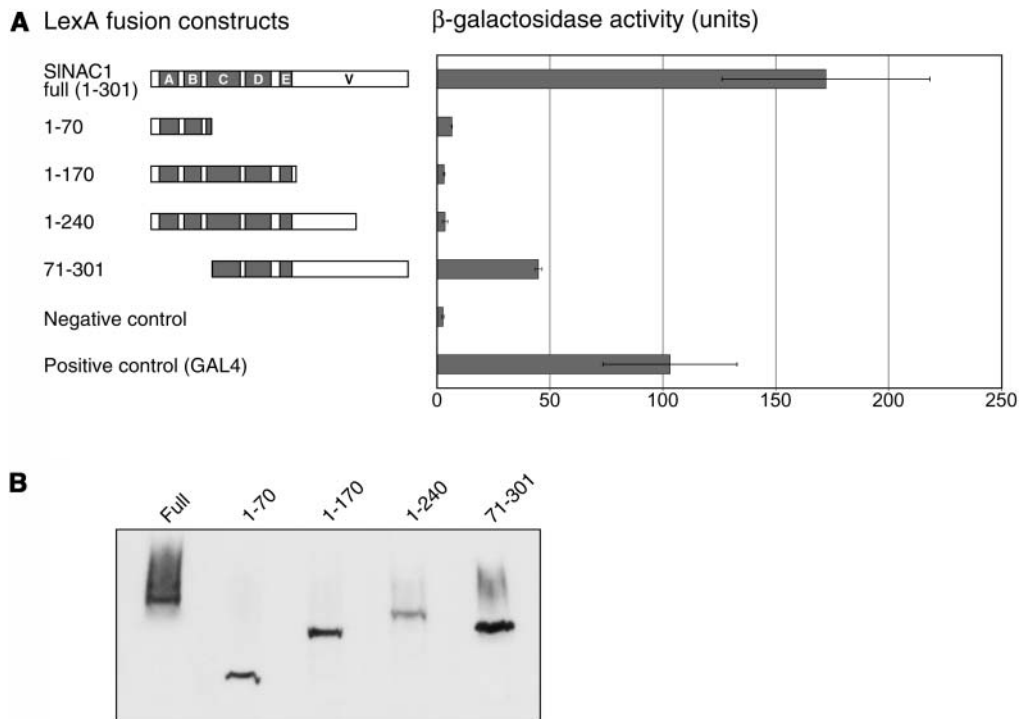


Figure 4. The Divergent C-Terminal Region of SINAC1 Is Able to Activate Transcription in Yeast.

(A) Regions of SINAC1 able to activate transcription in yeast. The LexA:SINAC1 fusion proteins are represented diagrammatically at left, with the positions of the NAC subdomains shown in shaded boxes (A to E) and the variable C terminus denoted V. The ability of LexA:SINAC1 fusion proteins to activate transcription in yeast is shown at right. Activities were assayed by measuring β -galactosidase activity in total protein extracts from cells containing pLexA-SINAC1 plasmids and pSH18-34, which contains a *lacZ* reporter gene downstream of the LexA recognition site. Positive control corresponds to yeast containing pSH18-34 and expressing a LexA fusion with the GAL4 AD. Negative control corresponds to yeast containing pSH18-34 and expressing LexA. Error bars indicate the standard deviation for each sample.

(B) Immunoblot analysis of yeast cells demonstrating that nontransactivating LexA:SINAC1 fusions are expressed at levels similar to those of transactivating fusion proteins. Total protein from yeast cultures containing fusion proteins was extracted, fractionated on SDS-polyacrylamide gels, and immunoblotted with anti-LexA.

located near its C terminus. Immunoblotting confirmed that all LexA:SINAC1 fusion proteins were produced at similar levels in yeast (Figure 4B).

In Vitro Binding of SINAC1 to TLCV REn

The specificity of the REn/SINAC1 protein interaction was tested using an in vitro pull down assay. A 6 \times His-REn fusion protein was expressed in *Escherichia coli*, purified to homogeneity, and mixed with crude soluble protein extracted from *E. coli* cells induced to express a SINAC1-calmodulin binding peptide (CBP) fusion protein containing a FLAG epitope (CBP-SINAC1). The mixture was incubated with nickel-nitrilotriacetic acid agarose (Ni-NTA), washed extensively to remove unbound protein, re-suspended in loading buffer, electrophoresed, and transferred to polyvinylidene fluoride (PVDF) membrane. The presence of CBP-SINAC1 and 6 \times His-REn was determined by immunoblotting using antibodies directed against FLAG and polyHis, respectively.

Bound CBP-SINAC1 was detectable when incubated with 6 \times His-REn (Figure 5, lane 5). To determine the specificity of

CBP-SINAC1 binding, it was added to Ni-NTA resin alone (lane 8) or in combination with purified 6 \times His-C2, another TLCV-encoded protein (lane 7). In both of these reactions, CBP-SINAC1 was not detected in the bound fraction, indicating that it was interacting specifically with 6 \times His-REn. To determine if 6 \times His-REn was specifically pulling down CBP-SINAC1, we mixed it with total soluble protein extracted from cells induced to express CBP-SIUPTG1, a control CBP-tagged protein (lane 6). No CBP-SIUPTG1 was detectable in the bound fraction, indicating that 6 \times His-REn does not indiscriminately bind abundant proteins in a mixture. SIUPTG1 is a tomato homolog of potato (*Solanum tuberosum*) UDP-glucose:protein transglucosylase identified in another of our yeast two-hybrid screens (accession number AY622990). All reactions were performed at least twice with consistent results.

REn and SINAC1 Are Targeted to the Nucleus

To investigate the potential role of SINAC1 in REn function in vivo and to further verify the putative interaction between these proteins, we examined the subcellular localization of SINAC1

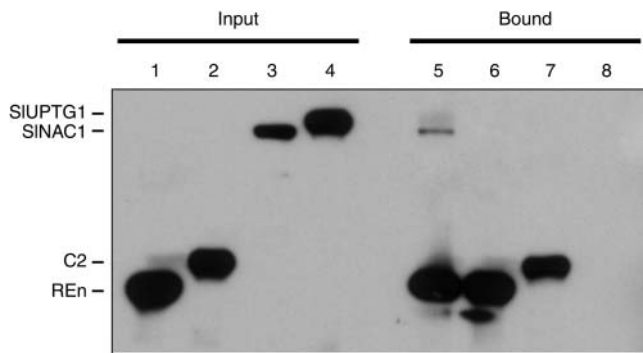


Figure 5. REn Interacts with SINAC1 in Vitro.

Purified 6×His-tagged proteins were mixed with crude CBP-tagged protein mixtures, incubated with Ni-NTA, and washed extensively to remove any unbound protein. Bound protein was resuspended in loading buffer, resolved by SDS-PAGE, and analyzed by immunoblotting using anti-polyHis and anti-FLAG (CBP-tagged proteins also contain a FLAG epitope) antibodies. Reactions were as follows: 6×His-REn and CBP-SINAC1 (lane 5), 6×His-REn and CBP-SIUPTG1 (lane 6), 6×His-C2 and CBP-SINAC1 (lane 7), and CBP-SINAC1 alone (lane 8). Protein inputs for each reaction are shown: 6×His-REn (lane 1), 6×His-C2 (lane 2), CBP-SINAC1 (lane 3), and CBP-SIUPTG1 (lane 4).

and REn in plant cells. Each of the open reading frames (ORFs) was fused to GFP downstream of the CaMV 35S promoter. The fusion proteins (REn:GFP and SINAC1:GFP) were transiently expressed in onion (*Allium cepa*) epidermal cells after biolistic delivery of vector DNA and analyzed by confocal microscopy.

Free GFP was distributed in both the cytoplasm and the nucleus of bombarded cells (Figure 6, bottom). By contrast, both REn:GFP and SINAC1:GFP localized exclusively to nuclei (top and second rows), which were clearly visible as dense ovoid structures when cells were viewed with differential interference optics (middle column). Further verifying this result, the distribution pattern of REn:GFP and SINAC1:GFP matched that of the Arabidopsis HISTONE 2B:yellow fluorescent protein (H2B:YFP) fusion protein (third row), a control for nuclear localization (Boisnard-Lorig et al., 2001). NAC1 from Arabidopsis (Xie et al., 2000) and CmNACP from pumpkin (*Cucurbita maxima*; Ruiz-Medrano et al., 1999) were also found to be nuclear proteins, implying that this is a general characteristic of NAC proteins and supporting the idea that they function as transcription factors. More importantly, however, the localization of REn and SINAC1 to the nucleus suggests that an opportunity exists for binding between these proteins in TLCV-infected plants.

TLCV Infection Induces the Expression of *SINAC1*

To analyze the endogenous expression of *SINAC1*, we performed an RNA gel blot analysis of total RNA preparations from tomato leaf tissue. *SINAC1* mRNA of the predicted size (~1300 nucleotides) was detectable at low levels in healthy tomato leaves (data not shown). To test whether *SINAC1* transcription might be regulated by TLCV infection, total RNA from new, emerging leaves of infected and healthy plants sampled at various time points postinoculation was analyzed (Figure 7A).

SINAC1 expression was strongly induced in infected plants at 10 d postinoculation (dpi) and maintained to at least 20 dpi, a result observed in three independent experiments. Infection with TYLCSV caused a similar increase in the levels of *SINAC1* transcript (Figure 7B), suggesting that induction of this gene is a general response to geminivirus infection. Some fluctuation in the level of *SINAC1* gene expression in healthy plants over the course of these experiments was also observed, although this was minimal compared with the induction caused by geminiviral infection.

We asked whether REn, given its physical interaction with SINAC1, plays a role in the regulation of *SINAC1* gene expression. Tomato leaf tissue was infiltrated with *Agrobacterium tumefaciens* cells harboring a REn expression construct (p35S-REn), and changes in *SINAC1* transcript accumulation were analyzed (Figure 7C). Expression of REn induced *SINAC1* gene expression to levels similar to that observed when tissue was infiltrated with cells containing a replicating TLCV construct. By contrast, tissue that was infiltrated with *A. tumefaciens* containing an empty expression vector or vectors designed to express two other TLCV-encoded genes, *C1* and *C2*, contained levels of *SINAC1* transcript similar to untreated tissue. These results suggested that induction of *SINAC1* in response to TLCV infection is mediated by REn, and also demonstrated that *SINAC1* is not induced nonspecifically in response to *A. tumefaciens* infection or wounding associated with the infiltration procedure. In a subsequent experiment, a TLCV derivative containing a mutation in the *C3* gene that prevents translation of the REn protein (Rigden et al., 1996) was tested for its effect on *SINAC1* expression. The level of *SINAC1* transcript in tomato plants agroinoculated with the REn mutant was comparable to healthy controls 25 dpi (Figure 7D, top). The presence of replicating REn mutant virus was confirmed by DNA gel blotting the same total nucleic acid samples and hybridizing with a TLCV-specific probe (Figure 7D, middle). Together, our results strongly imply that REn alone is responsible for induction of *SINAC1*. It must be noted that, as expected, the amount of viral DNA in extracts obtained from REn mutant-infected plants (middle, right lane) was much lower than equivalent samples from plants infected with wild-type virus (middle, lane M; the ratio of REn mutant:wild-type total nucleic acid extracts is 20:1). Thus, this experiment does not rule out the possibility that the absence of *SINAC1* induction in REn mutant-infected plants is because of reduced viral load.

In all RNA gel-blot analyses, indistinguishable results were obtained when membranes were hybridized with probes synthesized from the full-length *SINAC1* gene or from only the divergent 3' sequence (data not shown), indicating that variation in the expression of other putative NAC genes in response to TLCV infection or to transient REn expression was insignificant.

TLCV Replication Is Tissue Specific, and *SINAC1* Induction Occurs Only in TLCV-Infected Cells

SINAC1 upregulation may be a systemic stress response, or alternatively TLCV may act to specifically induce expression of this gene in infected cells. To distinguish between these possibilities, we performed in situ hybridization experiments to analyze the specific regions of *SINAC1* mRNA accumulation

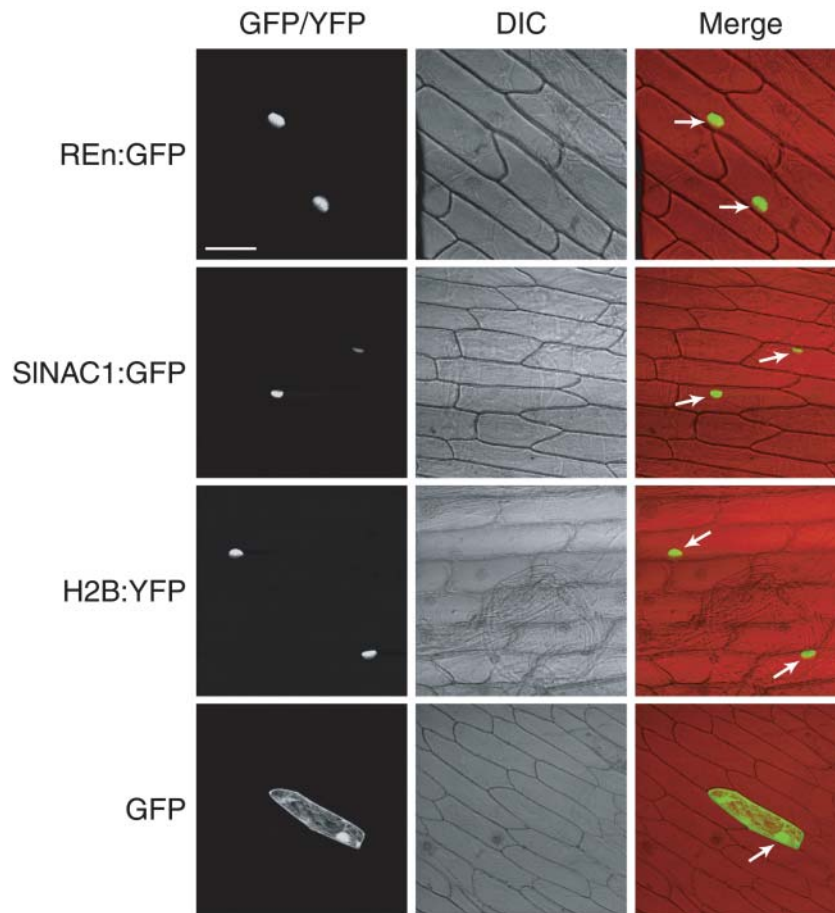


Figure 6. REn and SINAC1 Localize to the Nucleus of Onion Cells.

REn:GFP (top row) and SINAC1:GFP (second row), as well as GFP alone (bottom row), were expressed in onion epidermal cells using the CaMV 35S promoter after biolistic delivery of vector DNA. A positive control for nuclear localization, H2B:YFP, is also shown (third row). Cells were analyzed for GFP and YFP fluorescence (left column) by confocal microscopy. Differential interference contrast (DIC) images and merge images are shown in the middle and right columns, respectively. Nuclei in merge images are indicated by arrows. Bar = 100 μ m.

compared with sites of TLCV infection. Hybridization of tomato tissue with TLCV and SINAC1 probes produced only very weak chromogenic signals (data not shown). Because TLCV-derived nucleic acid accumulates to much higher levels in *Nicotiana benthamiana*, leaf tissue derived from this host was analyzed. A single-stranded RNA (ssRNA) complementary to the TLCV V2 gene produced a strong signal that was observed mainly in phloem cells but also in some xylem parenchyma and bundle sheath cells (Figures 8B and 8E). This indicates that TLCV is limited to vascular tissue, a characteristic also reported for *Abutilon mosaic virus*, *Squash leaf curl virus*, and *Tomato yellow leaf curl virus* from the Dominican Republic (Horns and Jeske, 1991; Sanderfoot and Lazarowitz, 1996; Rojas et al., 2001). No signal was obtained when healthy *N. benthamiana* leaf tissue was hybridized with a probe complementary to the divergent 3' SINAC1 sequence, which should not detect unrelated *N. benthamiana* NAC proteins (Figure 8A). However, in TLCV-infected sections, a SINAC1 homolog was detected in some phloem cells (Figures 8C and 8F). To test whether induction of this gene was

occurring only in cells infected with TLCV, dual-color in situ hybridizations were performed (Jowett, 2001). Hybridization of the TLCV probe to sections exhibiting a SINAC1 signal produced a distinctive purple chromogenic output (Figures 8D and 8G). This color is produced by the masking of the red SINAC1 signal by the blue viral signal, and confirms that almost every cell that accumulated substantial amounts of SINAC1 mRNA also contained TLCV. Thus, induction of a *N. benthamiana* SINAC1 homolog in response to TLCV infection is not a systemic response but rather is restricted to cells infected with the virus.

The Expression Level of SINAC1 Is a Determinant of Geminiviral Replication

To investigate the possible function of SINAC1 in relation to TLCV infection, the effect of constitutive, high-level expression of this gene on TLCV replication was analyzed. A transient TLCV replication system, based on *Agrobacterium*-mediated delivery of an infectious TLCV construct into *N. benthamiana* leaf strips

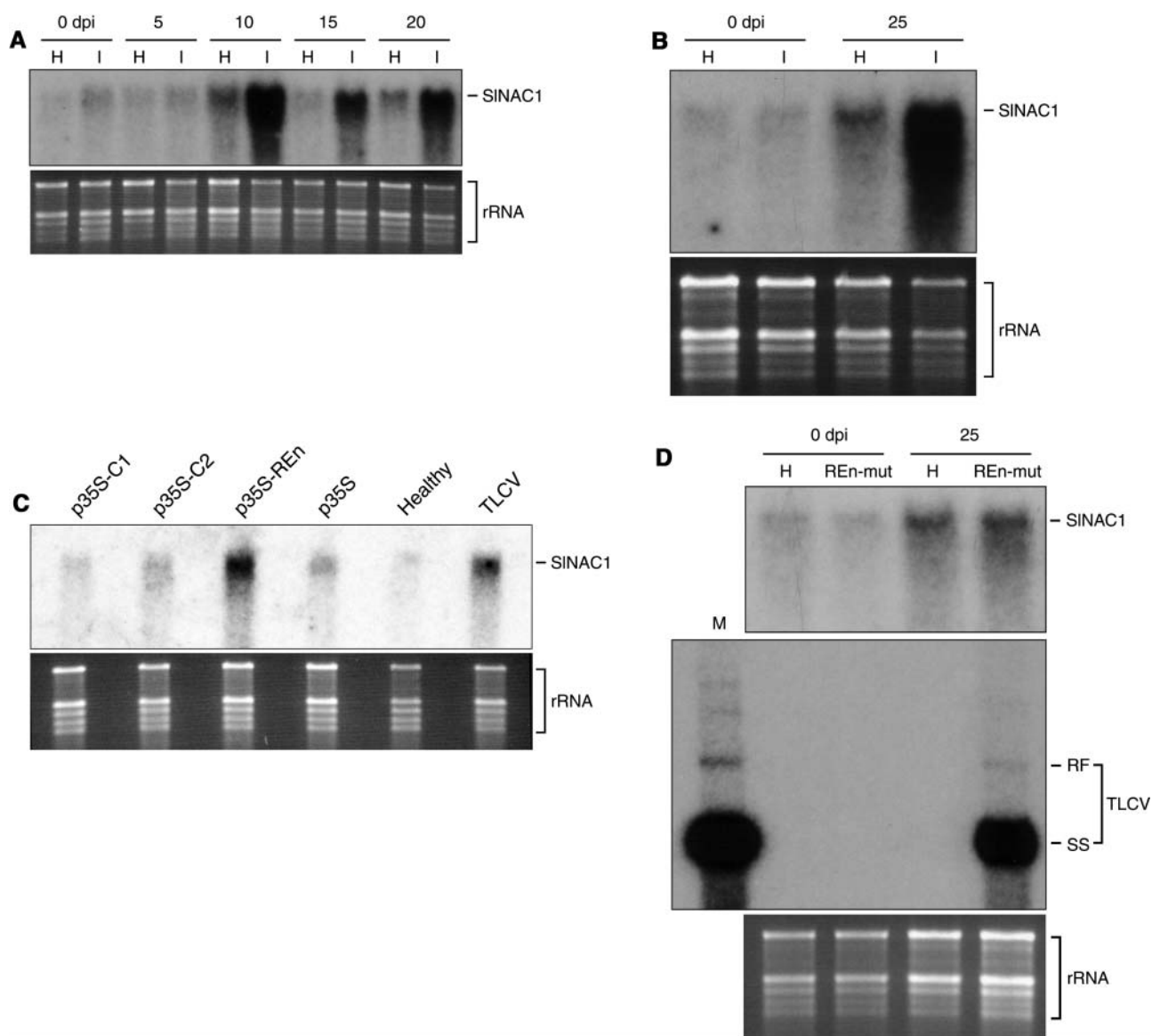


Figure 7. *SINAC1* Is Induced by TLCV Infection.

(A) TLCV infection results in an upregulation of *SINAC1* gene expression. RNA gel blot showing the expression of *SINAC1* in healthy (H) or TLCV-infected (I) tomato plants. Tissue samples were obtained at 0, 5, 10, 15, and 20 dpi.

(B) TYLCSV infection results in an upregulation of *SINAC1* gene expression. RNA gel blot showing the expression of *SINAC1* in healthy or TYLCSV-infected tomato plants. Tissue samples were obtained 0 and 25 dpi.

(C) Transient expression of *REn* is sufficient to induce *SINAC1* gene expression. Tomato leaves were infiltrated with *A. tumefaciens* cells containing a replication-competent TLCV 1.1mer, p35S, or p35S expressing the TLCV genes *C1*, *C2*, and *REn*. RNA was extracted from tissues 5 d postinfiltration and *SINAC1* expression analyzed by RNA gel blotting.

(D) A TLCV *REn* mutant cannot induce *SINAC1* gene expression. RNA gel blot showing the expression of *SINAC1* in healthy plants or plants infected with a TLCV *REn* mutant (*REn*-mut) at 0 and 25 dpi (top). The presence of replicating TLCV *REn* mutant was confirmed by DNA gel blotting (middle). In this blot, we also ran an extract obtained from plants infected with wild-type virus (left, designated M); the ratio of *REn* mutant:wild-type virus total nucleic acid extracts is 20:1. TLCV DNA species are marked RF (supercoiled double-stranded replicative form) and SS (single stranded).

(Dry et al., 1997), was used in this study. When *A. tumefaciens* cells harboring the infectious TLCV construct were combined with *A. tumefaciens* containing an *SINAC1* expression construct, TLCV ssDNA accumulated to a level equivalent to that observed in TLCV-infected plants (Figure 9A, cf. lanes 1 and 3). The level of

TLCV ssDNA produced in the presence of 35S-driven *SINAC1* expression was considerably more than that observed in our control treatment, in which TLCV was combined with an empty expression construct (lane 2). This response was observed in four independent experiments in which all treatments were

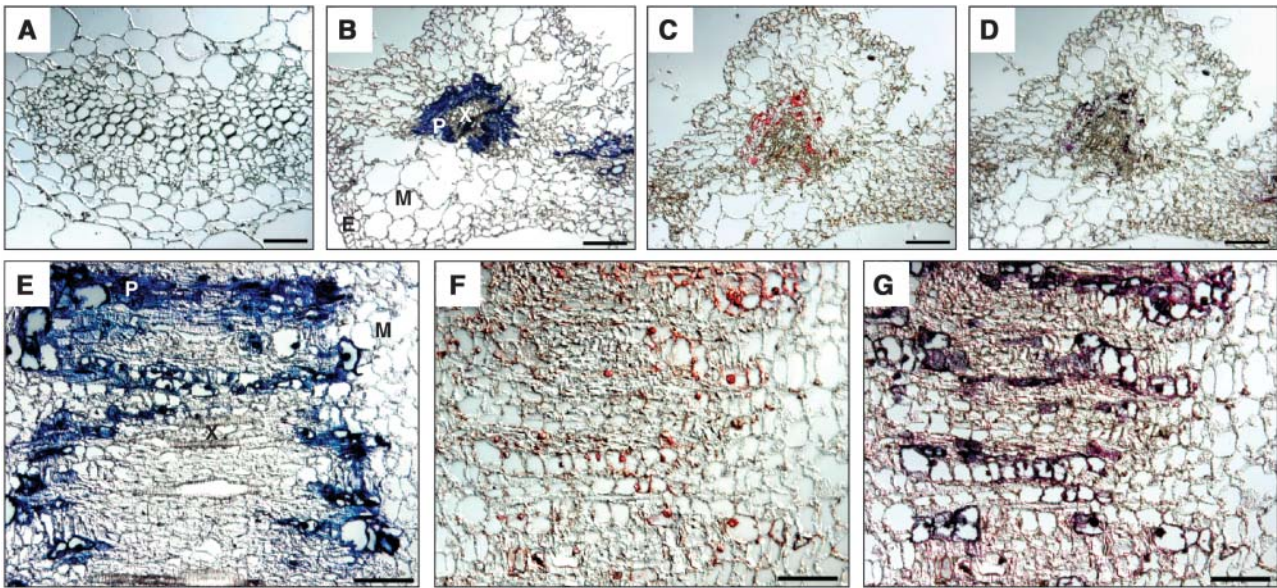


Figure 8. Induction of *SINAC1* by TLCV Occurs Only in Infected Cells.

Tissue sections derived from mock-inoculated (**A**) and TLCV-infected (**B**) to (**G**) leaves of *N. benthamiana* were hybridized with either fluorescein-labeled ssRNA probe complementary to *SINAC1* (**A**), (**C**), (**D**), (**F**), and (**G**) or DIG-labeled ssRNA probe complementary to TLCV (**A**), (**B**), (**D**), (**E**), and (**G**). (**A**) to (**D**) are cross sections and (**E**) to (**G**) are longitudinal sections taken from the main leaf vein. Bar = 100 μ m. Cell types present are indicated: E, epidermal; M, mesophyll; P, phloem; and X, xylem.

performed in duplicate. The level of *SINAC1* expression in all leaf strip samples was concurrently analyzed by semiquantitative real-time PCR, which confirmed that the enhancement of TLCV ssDNA accumulation was associated with *SINAC1* expression by p35S-*SINAC1* (Figure 9B). Together, these results suggest that *SINAC1* is involved in TLCV replication in planta.

DISCUSSION

Because of their limited coding capacities, geminiviruses depend on host factors to amplify their genomes. In quiescent cells that have exited the cell division cycle and cannot support DNA replication, these pathogens must therefore induce the required replicational machinery. To achieve this, they encode proteins that increase the expression level of growth-promoting genes and/or alter the function of cell-cycle regulatory proteins, often by physically interacting with host factors. For example, the geminiviral Rep protein upregulates expression of *PCNA*, possibly by interfering with Rb/E2F-mediated transcriptional repression of the *PCNA* gene through its interaction with Rb (Egelkrout et al., 2001). Rep also binds histone H3 (Kong and Hanley-Bowdoin, 2002), suggesting that it may act to alleviate repression of virus replication and transcription processes induced by the packaging of geminiviral double-stranded DNA species into minichromosomes (Abouzid et al., 1988; Pilartz and Jeske, 1992). The other viral protein required for high levels of viral DNA accumulation, REn, is involved in several protein-protein interactions. It binds to Rep and may increase the affinity of this protein for the viral origin of replication (Fontes et al., 1994; Settlege et al., 1996; Gladfelter et al., 1997), an activity proposed

to enhance viral replication (Hanley-Bowdoin et al., 1999). REn also interacts with the host Rb and PCNA proteins, suggesting that its role in replication is multifaceted (Settlege et al., 2001; Castillo et al., 2003). Consistent with this idea, we report here that a new tomato protein of the NAC domain family, *SINAC1*, is induced by and interacts with REn and appears to be involved in viral replication.

The Role of *SINAC1* in TLCV Infection

In a transient replication system, expression of *SINAC1* considerably enhanced the accumulation of TLCV ssDNA (Figure 9A), suggesting that this gene may facilitate TLCV replication. It is not logical to consider that tomato plants would retain a gene that promotes disease, and, therefore, *SINAC1* must perform some essential cellular function. However, our results suggest that geminiviruses, through the action of REn proteins, have hijacked the innate role of *SINAC1*. Several mechanisms can be envisioned to explain this result. One is that *SINAC1* acts indirectly in TLCV replication as a positive regulator of cellular genes required during viral infection. For example, it may activate transcription of genes required for S-phase functions that are normally absent in differentiated cells, a strategy analogous to the putative release of E2F transcription factors when geminiviral Rep proteins bind Rb. This explanation does not appear to correlate with the proposed function of other NAC proteins in meristem development and plant senescence pathways, where these factors contribute to a decision of cells to leave the proliferative state and take a certain differentiation pathway. For example, NAM is thought to interfere with cell division around the developing shoot

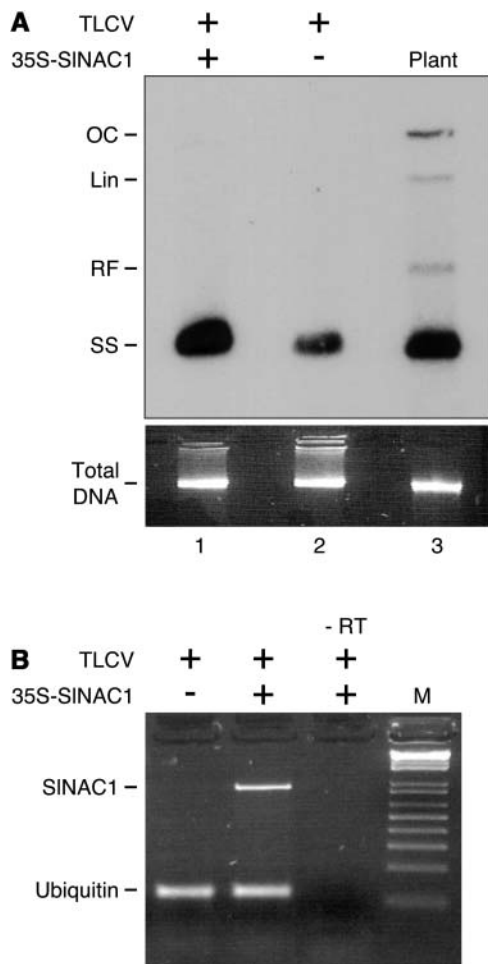


Figure 9. *SINAC1* Expression Enhances TLCV ssDNA Accumulation.

(A) Expression of *SINAC1* enhances TLCV ssDNA accumulation in a transient replication assay. *A. tumefaciens* cells harboring Bin19-TLCV1.1 were combined with *A. tumefaciens* cells containing either an empty expression construct (lane 2) or p35S-*SINAC1* (lane 1) and cocultivated for 48 h with leaf strips from *N. benthamiana* plants. DNA was extracted from tissue samples 3 d later and replication of TLCV analyzed by DNA gel blotting. Lane 3 (Plant) is a sample extracted from TLCV-infected *N. benthamiana* used as a marker for TLCV DNA forms, marked OC (open circular double stranded), Lin (linear double stranded), RF (supercoiled double-stranded replicative form), and SS (single stranded). OC, Lin and RF DNA forms were observed in extracts from *N. benthamiana* leaf strips after longer exposures.

(B) Analysis of *SINAC1* expression by p35S-*SINAC1* in *N. benthamiana* leaf strips by semiquantitative RT-PCR. Total RNA was prepared from leaf strips treated with TLCV plus an empty expression construct or TLCV plus p35S-*SINAC1*. Ubiquitin mRNA served as an internal control. RT reaction mix without reverse transcriptase was used as a negative control (marked -RT). M, size markers.

apical meristem (Souer et al., 1996) to drive flower development. Further, the observation that GRAB proteins from wheat interfere with replication of WDV led Xie et al. (1999) to speculate that these NACs play a role in the pathway leading to cell differentiation. However, the family of genes encoding NAC domains is

very large and members appear to possess highly diverse functions. Thus, it is reasonable to speculate that some NACs could upregulate genes involved in processes advantageous to geminivirus replication, such as DNA replication, transcription, or the G1/S transition of the cell cycle. Supporting this idea, *NAC1* from *Arabidopsis* is involved in the initiation of lateral root development (Xie et al., 2000), whereas *CUC1* promotes adventitious SAM formation by maintaining epidermal cells in an undifferentiated state in transgenic *Arabidopsis* (Takada et al., 2001; Hibara et al., 2003).

Another possibility is that *SINAC1* functions directly in geminiviral replication. There are numerous examples in which host transcription factors play an important and direct role in activating the DNA replication of mammalian oncoviruses by binding the viral origin of replication and increasing the initiation frequency (Li et al., 1998, and references cited therein). Alternatively, REn may recruit *SINAC1* into a DNA replication complex, where it could promote amplification of the viral genome. This idea is supported by the observation that REn interacts with PCNA (Castillo et al., 2003), a host factor that acts as a sliding clamp and modulates the interaction of other proteins, including polymerases, with DNA (reviewed in Hingorani and O'Donnell, 2000).

A third possible scenario is that *SINAC1* could positively modulate transcription of viral genes. Although the geminiviral C2 protein is responsible for activating virion-sense gene expression (Sunter and Bisaro, 1992; Sunter et al., 1994; Dry et al., 2000), expression of the complementary-sense genes is probably controlled by host factors. *SINAC1* mediates expression of a reporter gene in yeast (Figure 4), suggesting that it may function endogenously as a transcription factor and, therefore, positively modulate *cis*-acting promoter elements in the geminiviral genome.

Mechanism of TLCV-Mediated *SINAC1* Induction

SINAC1 gene expression was upregulated in response to TLCV infection (Figures 7 and 8). Two lines of evidence presented in this study support the idea that this induction is mediated by REn. First, transient delivery of a *REn* expression construct resulted in increased accumulation of *SINAC1* mRNA, whereas control constructs were unable to engender this response (Figure 7C). Second, a TLCV REn mutant was unable to upregulate *SINAC1* despite accumulating to moderate levels in infected tissue (Figure 7D). Several mechanisms by which *SINAC1* is induced can be envisioned. First, REn could act directly as a transactivator of *SINAC1* gene expression. Analysis of the peptide sequence of TGMV REn revealed that its acidic N terminus resembles some transcriptional ADs (Hanley-Bowdoin et al., 1999). Second, *SINAC1* induction may be a side effect of the presence of REn in a plant cell. It is doubtful that it occurs via the putative REn-Rb or REn-PCNA interactions because Rep, which also binds these host factors, was unable to stimulate *SINAC1* expression. However, *SINAC1* induction may occur because REn is impinging on other cellular processes, possibly through an as yet uncharacterized protein interaction. This explanation is supported by the observation that REn and Rep produced highly disparate phenotypic effects when transiently expressed in host plants (Selth et al., 2004). Finally, although at this time we have no

evidence to suggest that induction of *SINAC1* relies on the REn-SINAC1 protein interaction, this possibility cannot be discounted. For example, by sequestering SINAC1 through physical interaction, REn may relieve a negative feedback mechanism by which SINAC1 inhibits transcription of its gene. Such a function is not unprecedented: *AtWRKY6*, a member of the large WRKY family of plant-specific transcriptional regulators, is able to suppress its own promoter activity while positively influencing the expression of genes involved in senescence and pathogen defense (Robatzek and Somssich, 2002).

It was originally proposed that NAC proteins could be divided into three subfamilies (Kikuchi et al., 2000). More recently, Ooka et al. (2003) performed a more comprehensive phylogenetic analysis of known NACs and putative Arabidopsis and rice NACs and identified 18 subfamilies. Members of the so-called ATAF subfamily, identified in both studies, appear to share a conserved role in the response to stress. Genes belonging to this group are induced by wounding (Collinge and Boller, 2001), fungal infection (Collinge and Boller, 2001; Hegedus et al., 2003), bacterial infection (Mysore et al., 2002), insect damage (Hegedus et al., 2003), and cold shock (Hegedus et al., 2003). *SINAC1*, which also belongs to the ATAF subfamily (Figure 1B), is induced by TLCV (this study) and *Pseudomonas syringae* (Mysore et al., 2002) infection, suggesting that it may play a general role in stress responses. However, four lines of evidence support the idea that stimulation of *SINAC1* gene expression by TLCV is a specific response and that SINAC1 plays an active role in TLCV infection. First, neither *A. tumefaciens* infection nor wounding associated with the agroinfiltration procedure induced *SINAC1*. Second, induction of *SINAC1* by TLCV is restricted to infected cells and appears to be mediated by the REn protein. Third, SINAC1 interacts with the TLCV-encoded REn protein. Finally, over-expression of *SINAC1* enhances the accumulation of TLCV DNA species in a transient replication system.

NACs Are Involved in Other Viral Infections

Xie et al. (1999) found an interaction between the WDV RepA protein and two wheat NACs, GRAB1 and GRAB2. The N terminus (amino acids 1 to 208) of TLCV Rep shares 39.5% sequence identity with full-length WDV RepA. However, we were unable to detect binding between bacterially expressed TLCV Rep and SINAC1 in vitro (data not shown). Another apparent difference between the WDV-GRAB and TLCV-SINAC1 interactions is that, although expression of both *GRAB* genes was shown to interfere with WDV DNA replication in cultured wheat cells, *SINAC1* expression enhanced TLCV ssDNA accumulation in a transient replication assay. The distinct roles of GRABs and SINAC1 in geminivirus infection may again reflect the functional diversity that exists between members of the NAC domain family. Supporting the idea of NACs possessing diverse roles in viral pathogenesis, the NAC domain-containing Arabidopsis TIP protein is involved in the *Turnip crinkle virus* resistance response pathway by interacting with the *Turnip crinkle virus* coat protein (Ren et al., 2000). Alternatively, it could denote different DNA replication strategies used by the highly divergent dicot-infecting TLCV and monocot-infecting WDV. It would be useful to examine the effect WDV infection has on the expression level of *GRAB1*

and *GRAB2* to see whether, in contrast with the situation with TLCV and *SINAC1*, the virus downregulates these detrimental genes.

REn has previously been reported to physically interact with itself, Rep, Rb, and PCNA. Thus, it probably plays several roles in geminiviral infection, including the establishment of a cellular environment competent for DNA replication and an involvement in initiation of viral DNA replication. Despite its apparent multifunctionality, our results strongly imply that the mechanism by which REn increases viral ssDNA accumulation involves its interaction with SINAC1. We do not yet know at which stage REn/SINAC1 binding is involved in TLCV replication: it may have a direct role in this process or could simply be required to induce *SINAC1*, which in turn acts to facilitate TLCV replication.

Table 1. Oligonucleotide Primers Used in This Study

Name	Sequence
P1	5'-TT GAATTC GATTCACGCACAGGGGAACC-3' ^a
P2	5'-GGGG CTCGAG TTAATAAAAAATTAATTTTA-3'
P3	5'-AAGC CTCGAG TCATGTGAAGTCCAGGAA-3'
P4	5'-GG GAATTC AACTACAACCACGAC-3'
P5	5'-AAGC CTCGAG TCATGAGTCTAGTACATT-3'
P6	5'-GGG GAATTC AAGTATTAGATAGT-3'
P7	5'-GG GAATTC AACAAAGGAGCAAACGGA-3'
P8	5'-AAGC CTCGAG CAATACCCTCTTTTTC-3'
P9	5'-AAGC CTCGAG TCAATGCTTCTCAAGTGT-3'
P10	5'-GGG GAATTC TTTTTCTCACCAAGG-3'
P11	5'-TT GCGGCCGC TTAGTAAGGTTTTTGCAT-3'
P12	5'-AAAAG GCGGCCGC TCAACATGGCGACAAGAC-3'
P13	5'-TTT GGATCC GAT TCACGCACAGGGGA-3'
P14	5'-GGGGGGGG GAAGCT TTTAATAAAAAATTAATTTT-3'
P15	5'-TT GGATCC CAGAATTCATCACCC-3'
P16	5'-GGG GAAGCT TTTAATACCCTCAAG-3'
P17	5'-TTT GGATCC AACAAAGGAGCAAACGGA-3'
P18	5'-TT GCGGCCGC TTAGTAAGGTTTTTGCAT-3'
P19	5'-TT GAATTC GCAGCAGCAACACCA-3'
P20	5'-GG CTCGAG CTACTTTTTAGTCTT-3'
P21	5'-GGGG TCTAGAT TAATAAAAAATTAATTTTA-3'
P22	5'-GGG TCTAGAT TAGTAAGGTTTTTGCAT-3'
P23	5'-TCC CCGGG GATGACTAGACCAAAGTCATCCGTAT- AAATGCTAA-3'
P24	5'-CG GATCCT CAATTCCTTCTCCTCCGGATGG-3'
P25	5'-GGG GATCC ATGTTCCAGAATTCATCACCCCTCAAC-3'
P26	5'-GGG GATCC TAAATACCCTCAAGAAACG-3'
P27	5'-CGG GTACC ATGGATTCACGCACAGGGGAACC-3'
P28	5'-TG CTCTAG AGTTAATAAAAAATTAATTTTATAT- CATGAT-3'
P29	5'-AAT CTAG AGAATTCAGTACCGCTCGCCAACG-3'
P30	5'-GGG GATCC GGTACCTTAGTAAGGTTTTTGCAT-3'
P31	5'-TT GGATCC AGCAAGCGACCAGCAGAT-3'
P32	5'-GGGG GAAGCT TTTAATTCCTGAATCGAATC-3'
P33	5'-CGG GTACC ATGAACAAAGGAGCAAACGGA- AATCAG-3'
P34	5'-TG CTCTAG ATTAGTAAGGTTTTTGCATGTATAGG-3'
P35	5'-CGGCATGCTTAACACATGCA-3'
P36	5'-AGCCGTTTCCAGCTGTTGTTTC-3'

^aSequences shown in boldface correspond to specific restriction enzyme sites.

However, the data presented here suggest that tomato plants silenced for *SINAC1* expression, if not impaired in other functions, may exhibit tolerance to TLCV infection. We are currently attempting to generate transgenic tomato plants stably expressing a *SINAC1*-hairpin construct to test this resistance strategy.

METHODS

Oligonucleotides Used in This Study

Oligonucleotide sequences shown in Table 1 were synthesized by GeneWorks (Adelaide, Australia). Bold letters in the oligonucleotide sequences indicate added restriction sites used for cloning.

Yeast Two-Hybrid Screen

Yeast (*Saccharomyces cerevisiae*) strain displayYEAST-L (*MAT α* , *trp1*, *his3*, *ura3*, *leu2::2 LexAop-LEU2*; Display Systems Biotech, Vista, CA), containing a *Leu* biosynthesis gene downstream of the DNA recognition sequence for the LexA DNA BD, was used in two-hybrid screening. The *REn* ORF was amplified by PCR using P1 and P2. The product was digested with *EcoRI* and *XhoI* and ligated into similarly digested pLexA (*HIS3* marker) to generate pLexA-*REn*, which expresses a fusion of *REn* to the LexA DNA BD. Yeast was first transformed with displayREPORTER (*URA3* marker), a vector containing the *GFP* ORF downstream of the DNA recognition sequence for the LexA DNA BD. Cells were then sequentially transformed with pLexA-*REn* and with a pJG4-5 (*TRP1* marker) tomato (*Solanum lycopersicum*) Rio Grande cDNA library (Zhou et al., 1995). Colonies were selected on agar plates lacking uracil, His, Trp, and *Leu* but containing galactose and raffinose to induce the *GAL1* promoter driving expression of the tomato cDNAs fused to the B42 AD. Large colonies appearing within 5 d and exhibiting *GFP* expression were spread on plates lacking uracil, His, and Trp, and then transferred back to plates selecting for activation of the *LEU2* gene to remove false positives. Cells were then grown in media lacking Trp to select for pJG4-5 and yeast plasmid DNA purified using an RPM yeast plasmid isolation kit (Q-Biogene, Carlsbad, CA). *Escherichia coli* KC8 was transformed with purified pJG4-5 plasmid DNA because this strain is *trp1*⁻ and its defect can be complemented by the *TRP1* gene present in pJG4-5. To further eliminate false positives, plasmid DNA purified from KC8 was transformed back into displayYEAST-L containing displayREPORTER and pLexA-*REn* and the activation of *GFP* and *LEU2* reassessed.

To analyze the domains of the proteins responsible for their interaction, truncations of the *REn* and *SINAC1* genes were cloned into pLexA and pJG4-5, respectively. The fragments amplified were as follows: *REn* encoding amino acids 1 to 70 (P1 and P3), *REn* 40-120 (P4 and P5), *REn* 90-134 (P6 and P2), *SINAC1* 1-70 (P7 and P8), *SINAC1* 1-170 (P7 and P9), and *SINAC1* 71-301 (P10 and P11). Products were digested with *EcoRI/XhoI* (*REn* 1-70, *REn* 40-120, *REn* 90-134, *SINAC1* 1-70, and *SINAC1* 1-170) or *EcoRI/NotI* (*SINAC1* 71-301) and ligated into similarly digested pLexA or pJG4-5.

The *SINAC1* truncation sequences described above were transferred into pLexA to delineate the protein's putative transcriptional AD in yeast. Two other *SINAC1* sequences were cloned into *EcoRI/NotI*-digested pLexA for this yeast one-hybrid study: full-length *SINAC1* (amplified using primers P7 and P11) and a fragment encoding amino acids 1 to 240 (P7 and P12).

To measure the ability of different LexA-*SINAC1* fusion proteins to act as transcriptional activators, a reporter plasmid that contains eight LexA operators that direct transcription of the *lacZ* gene was used (pSH18-34; Golemis et al., 1994). Quantitative β -galactosidase assays from liquid cultures were performed according to the Yeast Protocols Handbook 2001

(Clontech, Palo Alto, CA) using *o*-nitrophenyl- β -D-galactopyranoside as substrate. A Microplate Reader 450 (Bio-Rad, Hercules, CA) was used to measure accumulation of the *o*-nitrophenol product. One unit of β -galactosidase is defined as the amount of activity hydrolyzing 1 nmol *o*-nitrophenyl- β -D-galactopyranoside per minute per cell. The assay was performed twice using three independent transformants for each construct. The positive control plasmid used in this study, pSH17-4, expresses a LexA fusion to the *GAL4* AD (Golemis et al., 1994).

To monitor fusion protein production in yeast, total protein (0.3 mL of yeast culture equivalent) was extracted and size fractionated on 4% to 20% Tris-Gly-SDS polyacrylamide gels (Gradipore, Frenchs Forest, Australia). Electrophoresed protein samples were transferred to Immobilon P PVDF membrane (Millipore, Billerica, MA) and blocked with 5% (w/v) nonfat dry milk before incubation with either rabbit anti-LexA polyclonal antibody (Invitrogen, Carlsbad, CA) to detect LexA-fusion proteins or anti-HA monoclonal antibody (Sigma-Aldrich, St. Louis, MO) to detect B42-fusion proteins. Donkey anti-Rabbit (Sigma) or Goat anti-Mouse IgG-horseradish peroxidase conjugate (Promega, Madison, WI) was used as the secondary antibody and detected using Super-Signal West Pico chemiluminescent substrate (Pierce Biotechnology, Rockford, IL).

Production of Recombinant Proteins and In Vitro Binding Experiments

Production of 6 \times His-tagged *REn* and C2 proteins was achieved using the pQE30 vector (Qiagen, Clifton Hill, Australia). The coding region of *REn* was amplified using oligonucleotides P13 and P14, digested with *BamHI/HindIII*, and ligated into similarly digested pQE30 to generate pQE30-*REn*. pQE30-C2 was constructed in the same way, using oligonucleotides P15 and P16 to amplify the C2 gene.

A 6 \times His-*REn* recombinant protein was purified using a protocol developed by Behjatnia et al. (1998) for the preparation of 6 \times His-Rep protein, with minor modifications. Briefly, *E. coli* M15 cells were transformed with pQE30-*REn*, grown to an OD of 0.9, and induced with 1 mM isopropyl- β -D-galactopyranoside (IPTG) for 3 h at room temperature. Cells were harvested, resuspended in Ni-NTA binding buffer (50 mM NaH₂PO₄, pH 8.0, 300 mM NaCl, 10 mM β -mercaptoethanol, and 1% Tween-20), and lysed by 1 mg/mL lysozyme, freeze-thawing, and sonication. Crude soluble protein was retrieved by centrifugation and 6 \times His-*REn* protein purified using Ni-NTA agarose (Qiagen). For the production of 6 \times His-C2, cells were transformed with pQE30-C2, grown to an OD of 0.7, and induced with 1 mM IPTG for 3 h at 37°C. Total soluble protein was extracted using sarkosyl by the method of Frangioni and Neel (1993) and dialysed against Ni-NTA binding buffer before purification of the recombinant protein.

Production of CBP-tagged *SINAC1* and *SIUPTG1* proteins containing a FLAG epitope was achieved using the pCAL-n-FLAG vector (Stratagene, La Jolla, CA). The coding region of *SINAC1* was amplified by PCR using the oligonucleotides P17 and P18, digested with *BamHI*, and ligated into *BamHI/SmaI*-digested pCAL-n-FLAG vector to generate pCAL-*SINAC1*. The *SIUPTG1* ORF was amplified using the oligonucleotides P19 and P20, digested with *EcoRI/XhoI*, and ligated into similarly digested pCAL-n-FLAG to yield pCAL-*SIUPTG1*.

E. coli B834-pLysS cells were transformed with pCAL-*SINAC1* and pCAL-*SIUPTG1*, grown to an OD of 0.7, and induced with 0.5 mM IPTG at 37°C for 3 h. Crude soluble protein was extracted using sarkosyl as described by Frangioni and Neel (1993).

Binding experiments were performed by adding 50 ng of a purified 6 \times His-tagged protein and 200 ng of total soluble protein extracted from cells induced to express the CBP-tagged protein of interest to 10 μ L of Ni-NTA agarose in 300 μ L of binding buffer (50 mM NaH₂PO₄, 300 mM NaCl, 20 mM imidazole, pH 8.0) in an Eppendorf tube. Tubes were then mixed

gently on a rotating platform at 4°C for 40 min. The resin was washed three times by brief centrifugation and resuspension in 400 μ L of binding buffer, resuspended in 50 μ L of sample loading buffer (0.5 M Tris-HCl, 10% glycerol, 2% SDS, 5% β -mercaptoethanol, and 1% bromophenol blue), and incubated at 94°C for 10 min. Aliquots (10 μ L) of eluate from the pelleted beads were size fractionated on 4 to 20% Tris-Gly-SDS polyacrylamide gels. Electrophoresed protein samples were transferred to Immobilon P PVDF membrane and blocked with 5% (w/v) nonfat dry milk before incubation with mouse anti-polyHis and anti-FLAG monoclonal antibodies (Sigma). Goat anti-Mouse IgG-horseradish peroxidase conjugate was used as the secondary antibody and detected using SuperSignal West Pico chemiluminescent substrate.

Analysis of GFP Fusion Proteins by Microprojectile Bombardment

A variant of the shuttle vector pART7 (Gleave, 1992) termed pART7-C'gfp, which contains the full-length GFP ORF (lacking the stop codon) upstream of the multiple cloning site (T. Franks, unpublished data), was used to transiently express REn:GFP and SINAC1:GFP fusion proteins in onion (*Allium cepa*) tissue. Full-length REn was amplified using primers P1 and P21, and the SINAC1 ORF was amplified using primers P7 and P22. After restriction enzyme digestion with EcoRI and XbaI, fragments were ligated into similarly digested pART7-C'gfp to generate C-terminal fusions with GFP. Also used in this experiment were pART7-ATG:GFP, which expresses free GFP (T. Franks, unpublished data), and pBI121-H2B:YFP, which expresses Arabidopsis H2B fused to the GFP yellow variant YFP (Boisnard-Lorig et al., 2001).

Onion epidermal strips on agar containing MS salt mixture (Invitrogen) were bombarded with each of the vectors. For four shots, 400 μ g of gold particles in 100 μ L of ethanol were vortexed for 2 min, spun down for 10 s in a microfuge, drained, washed twice with sterile water, and resuspended in 25 μ L of 40% glycerol. While gently vortexing, 4 μ L of the plasmid solutions (400 ng/ μ L), 10 μ L of cold 0.1 M spermidine, and 25 μ L of 2.5 M CaCl₂ were added dropwise and the resulting mixture incubated on ice for 10 min. The particles were spun down, washed with 70% ethanol, resuspended in 24 μ L of cold 100% ethanol, and 6- μ L aliquots were placed onto sterile filter holders. After bombardment, tissue was stored in the dark for 48 h and GFP/YFP expression visualized using a Bio-Rad Radiance 2100 confocal laser scanning microscope system. The excitation wavelength used for both GFP and YFP analysis was 488 nm.

Analysis of SINAC1 Gene Expression

Three-week-old tomato plants were inoculated with TLCV, TYLCSV, or the TLCV REn mutant (Rigden et al., 1996) using *Agrobacterium tumefaciens* (Grimsley et al., 1987). Total nucleic acid was extracted at various time points from new, emerging leaves and subjected to RNA gel blot analysis as described (Selth et al., 2004). To detect replication of the REn mutant, the same samples were analyzed by DNA gel blotting as described (Dry et al., 1993).

The binary vector pART27 was used to express individual TLCV genes to analyze their effect on SINAC1 mRNA production. Primers to amplify C1 (P23 and P24), C2 (P25 and P26), and C3 (P27 and P28) were designed. The PCR products were digested with *Sma*I/*Hind*III (C1), *Bam*HI (C2), and *Kpn*I/*Xba*I (C3) and ligated into similarly digested pART7. Fragments containing the CaMV 35S promoter upstream of the TLCV gene were liberated from these plasmids by digestion with *Not*I and ligated into pART27 previously cut with *Not*I and dephosphorylated. The resultant vectors were designated p35S-C1, p35S-C2, and p35S-C3. Young tomato leaves were infiltrated with *A. tumefaciens* C58 cells containing the p35S constructs as described (Selth et al., 2004). At 5 d postinfiltration, SINAC1 expression was analyzed by RNA gel blot analysis.

In Situ Hybridizations

Templates for the generation of ribonucleic probes were constructed as follows. A fragment comprising nucleotides 401 to 906 of SINAC1 was amplified using primers P29 and P30 and ligated into pGEM-T-Easy (Promega). The full-length TLCV V2 ORF was amplified using primers P31 and P32 and ligated into pGEM-T-Easy. Plasmids were linearized with *Nde*I (SINAC1) or *Sa*II (V2) and transcribed with T7 RNA polymerase. RNA probes labeled with fluorescein-12-UTP (for SINAC1 probes) and digoxigenin (DIG)-11-dUTP (for TLCV probes) were prepared using fluorescein or DIG RNA labeling mix, respectively (Roche Diagnostics, Castle Hill, Australia).

Plant material was collected from TLCV-infected plants 3 weeks postinoculation. Preparation of tissue sections and hybridization of DIG- and fluorescein-labeled probes was performed as described by Guerin et al. (2000). Probes were detected using Fast Red (Roche Diagnostics) or Western Blue substrates (Promega). For dual-color in situ hybridizations, probes were applied simultaneously and detected sequentially (Jowett, 2001).

Analysis of TLCV DNA Replication

The vector pART27 (Gleave, 1992) was used to transiently overexpress SINAC1 in *Nicotiana benthamiana* leaf strips. The entire SINAC1 ORF was amplified by PCR using primers P33 and P34, digested with *Kpn*I and *Xba*I, and ligated into *Kpn*I/*Xba*I-digested pART7. A DNA fragment containing the CaMV 35S promoter and the SINAC1 ORF was released by *Not*I digestion and ligated into similarly digested pART27 to generate p35S-SINAC1. *A. tumefaciens* strain C58 was transformed separately with p35S-SINAC1, empty pART27, and a Bin19 construct containing a TLCV 1.1mer (Bin19-TLCV1.1; Rigden et al., 1996). Cultures were grown at 28°C for 48 h and used in leaf strip transient replication assays as described (Dry et al., 1997). *A. tumefaciens* containing Bin19-TLCV1.1 was cocultivated with leaf strips in combination with *A. tumefaciens* harboring empty pART27 or p35S-SINAC1 at a ratio of 1:2. Viral replication in agroinoculated tissues was analyzed by DNA gel blotting as described (Dry et al., 1993).

Quantitation of SINAC1 mRNA Expression by Semiquantitative Reverse Transcription-PCR

Total RNA from *N. benthamiana* leaf strips was prepared using an RNeasy plant mini kit (Qiagen), which includes a treatment with RNase-free DNase. Semiquantitative reverse transcription (RT)-PCR was performed using a SuperScript one-step RT-PCR kit (Invitrogen) and 80 ng of RNA as template. The SINAC1 primers (P33 and P34) were used at a final concentration of 0.2 μ M. The internal control, ubiquitin, was amplified with primers P35 and P36 (Jin et al., 2002) used at a final concentration of 0.05 μ M. RT reaction mix without reverse transcriptase served as a negative control. After the linear phase of DNA amplification (26 cycles), the PCR products were examined by electrophoresis in a 2.0% agarose gel.

Sequence data from this article have been deposited with the EMBL/GenBank data libraries under accession numbers AY498713 (SINAC1) and AY622990 (SIUPTG1).

ACKNOWLEDGMENTS

We thank Mandy Walker, Ian Dry, Ming-Bo Wang, and Masumi Robertson for helpful discussions and careful reading of the manuscript. We are indebted to Anna Koltunow and Susan Johnson for insight regarding the in situ experiments, Jamus Stonor for excellent technical assistance, Ghafar Sarvestani for assistance with the confocal scanning laser microscope, Jim Haseloff for supplying pBI121-H2B:GFP, and

Gregory Martin for the gift of the tomato cDNA library. S.D. was supported by Australian Research Council Grant A09802106.

Received August 25, 2004; accepted October 18, 2004.

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