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The Nuclear Localization of the Arabidopsis Transcription Factor TIP Is Blocked by Its Interaction with the Coat Protein of *Turnip Crinkle Virus*

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Introduction

The interactions between a pathogen and its plant host occur at multiple levels to determine the outcome of the infection. In the case of a compatible interaction that ultimately leads to plant disease, the pathogen colonizes the host successfully because the host defenses have been compromised in some manner. In an incompatible interaction, the pathogen undergoes more limited self-propagation before an effective host defense is activated. This usually leads to a hypersensitive response (HR) at the initial foci of infection and systemically acquired resistance (SAR) in the whole plant (Baker et al., 1997). This HR-mediated resistance response has long been recognized as being under the control of a single resistance gene (R gene) in the host plant (Flor, 1971) and a corresponding avirulence gene (avr gene) in the pathogen.

The molecular characterization of numerous plant R genes and their corresponding pathogen avr genes (Marathe and Dinesh-Kumar, 2003) has suggested that the original receptor-ligand model (Gabriel and Rolfe, 1990), which postulated that physical recognition of avr gene product by the corresponding R gene product initiated the resistance cascade, was an oversimplification of the molecular events initiating the resistance response because the avr gene products were rarely found to directly interact with the R gene-coded counterparts. In several cases, additional host proteins, not the R protein directly, have now been identified that interact with the avr gene products (now more appropriately termed as effectors), and it...
is these secondary interactions that initiate the resistance cascade (Mackey et al., 2002, Mackey et al., 2003, Ren et al., 2000, Shao et al., 2003). It has also been shown that these novel host proteins are usually present in both susceptible and resistant hosts, which may explain why the effector proteins frequently cause more severe symptoms in susceptible plants than their nonresistance-eliciting variants (Abramovitch et al., 2003).

These findings have prompted the introduction of a new model, the guard hypothesis, to account for the early recognition events that lead to the resistance response in R gene-mediated host-pathogen interactions (Dangl and Jones, 2001, van der Biezen and Jones, 1998). The guard hypothesis proposes that effector proteins debilitate host defenses by either interfering with or eliminating one or more key components of basal defense pathways. The function of R proteins is to guard these host components and to mount a resistance response once changes in these host factors are detected. The Arabidopsis thaliana protein RIN4 is an elegant example of one such proposed basal defense factor. It has been shown to be targeted by three different effector proteins produced by the bacterial pathogen Pseudomonas syringae, and it is involved in the resistance responses mediated by two different R genes (Axtell and Staskawicz, 2003, Mackey et al., 2002, Mackey et al., 2003).

In the process of studying the interaction between Turnip crinkle virus (TCV) and Arabidopsis, we identified a host protein TIP that interacts specifically with TCV coat protein (CP) in a yeast two-hybrid screen (Ren et al., 2000). It was suggested that TIP might be involved in the Arabidopsis resistance to TCV in a manner consistent with the guard hypothesis because the CP is the effector protein that elicits the resistance response to TCV mediated by the HRT gene in A. thaliana ecotype Di-17 (Cooley et al., 2000). We further demonstrated that CP mutants unable to interact with TIP were also unable to elicit the HRT-mediated resistance response in Di-17, thus potentially implicating TIP in the resistance response, perhaps as a novel basal resistance factor. We also found that TIP activates the transcription of reporter genes in yeast cells and that it is a member of the NAC family of putative transcription factors (Duval et al., 2002, Riechmann et al., 2000, Ruiz-Medrano et al., 1999, Souer et al., 1996, Xie et al., 2000), suggesting a functional role as a transcription factor.

In this report, we have quantified the levels of physical interaction between TIP and wild-type and mutant forms of the CP unable to elicit the resistance response. We have further evaluated the role of TIP in gene transcription by examining its DNA-binding and nuclear localization properties. Our results confirm that TIP is most likely a transcription factor. More importantly, our results demonstrate that TCV CP prevents nuclear localization of TIP when both are co-expressed in plant cells. These data point toward a model in which TIP acts as a transcriptional activator of an as yet to be identified anti-viral basal resistance pathway. Our model is consistent with the prevalent guard hypothesis whereby the viral CP has evolved the ability to interfere with TIP and downregulate basal resistance. The host has countered this strategy by surveilling the attack on basal defenses with the HRT protein (Cooley et al., 2000).

Results

The TIP–CP physical interaction is verified with an in vitro binding assay

We have previously shown that TCV CP interacts with TIP in yeast cells and that the TIP-interacting region in the CP mapped to the N-terminal 25 amino acids (AA) of its RNA-binding domain (R domain). We further demonstrated in that study (Ren et al., 2000) that five CP mutants containing single amino acid (AA) changes within the R domain (N3A, D4N, P5A, R6A, and D13A) lost the interaction with TIP in yeast cells and viruses containing these five mutations also lost the ability to trigger the resistance response in A. thaliana ecotype Dijon-17. Conversely, the one CP mutant (G14A) that retained the ability to interact with TIP also retained the ability to trigger the resistance when incorporated back into the virus (Ren et al., 2000). These results suggested that the CP–TIP interaction is correlated with the ability of CP to elicit the resistance response. To confirm this correlation, we felt it important to assess the binding ability of wild-type and mutant CP with TIP in vitro. To demonstrate the interaction in vitro, we produced a fusion protein in which TIP was fused to the C-terminus of glutathione-S-transferase (GST-TIP, see Experimental procedures). Wild-type and mutant CPs were then radiolabeled with 35S through in vitro transcription. The GST-TIP was first immobilized on glutathione-sepharose beads, followed by the addition of the 35S-labelled TCV CP or mutant CP. After extensive washing to remove the unbound radioactivity, the beads were subjected to SDS-PAGE analysis to reveal any CP derivatives bound to GST-TIP. The GST protein was also produced and included in every experiment as a negative control.

We first tested the in vitro binding between TIP and full-length TCV CP. As shown in Figure 1A, lanes 1–3, GST-TIP retained a significant portion of 35S-labeled TCV CP. As expected, the GST control did not. An equivalent amount of the in vitro-translated CP was loaded on the gel for comparison (lane 1, unprecipitated). Note here that the lane with in vitro-translated TCV CP (lane 1) contained multiple smaller bands that are likely degradation products. However, only the full-length CP was bound to GST-TIP (lane 3). We then tested portions of the R domain for their ability to bind to GST-TIP, including the entire R domain (R), the N-terminal half of the R domain (RN, 25 AA), and the remaining portion of the R domain (RC, 27 AA). The results show that the R domain alone was able to bind efficiently to GST-TIP (Figure 1A, lane 12). It was also evident that the N-terminal 25 AA region bound more weakly than the entire R domain (lane 6) and
that the C-terminal portion of R domain bound poorly if at all (lane 9). These results correlated well with previous data derived from yeast two-hybrid assays. In this initial experiment, we also included the R domain of one of the resistance-breaking CP mutants (N3A) that was no longer able to interact with TIP in yeast cells. It displayed a markedly reduced TIP-binding ability compared to the wild-type R domain (compare lanes 12 and 15).

We further examined additional single AA substitution mutants within the N terminus of CP and the results are shown in Figure 1B. Among these mutants, all except R8A and F10A were reported previously (Ren et al., 2000). The R8A and F10A mutants behaved similarly to the other resistance breaking R domain mutants in that they were unable to interact with TIP in yeast cells and could not infect Di-17 plants systemically (unpublished data). It is clear from these results that for each of the resistance breaking CP mutants, their corresponding R domains bound to TIP significantly more weakly than did the wild-type R domain. Only the G14A mutant, which interacted with TIP in yeast cells and elicited a resistance response in Di-17, bound to TIP with similar affinity as the wild-type R domain. We conclude from these data that there is a strong positive correlation between CP–TIP interaction in yeast cells and the degree of physical binding in vitro. Clearly, the inability of most of CP mutants to interact with TIP in yeast cells was reflected as lower binding affinity in vitro.

To quantitatively evaluate the difference in binding affinity between various CP mutants and wild-type CP, the binding experiments were repeated four times using the R domains of wild-type CP and three representative mutants (R6A, R8A, and G14A). The X-ray films were then scanned using a densitometer and the relative amount of mutant R domains bound to GST-TIP was determined by comparing with the wild-type CP R domain bound (100%). The results presented in Figure 1C demonstrate that the wild-type R domain and G14A displayed 5-fold higher TIP-binding capacity than the R6A and R8A mutants. Together, the data presented in this section validate the physical interaction between TIP and TCV CP and confirm the requirement of an intact wild-type R domain for this interaction.

The C-terminal 100 AA region of TIP is required for interaction with TCV CP and the N-terminal 268 AA of TIP is sufficient for transcriptional activation

We next wanted to map the functional domains of TIP in an effort to elucidate its role in the plant resistance pathway targeting TCV. We learned previously that TIP had two primary activities: interaction with TCV CP and activation of reporter gene transcription in yeast cells (Ren et al., 2000). To delineate the region housing the transcriptional activation domain, regions of the TIP gene were fused to the GAL4 DNA-binding domain (DBD) of the yeast shuttle vector pAS2-1 and assayed for their ability to activate the LacZ reporter gene in a yeast one-hybrid assay. These same regions of the TIP gene were also fused to the GAL4 activation domain (AD) of pGAD10 to determine the region of TIP responsible for interacting with CP in a yeast two-hybrid assay. The results of these experiments are summarized in Figure 2. The ability of TIP to activate transcription was retained in the N-terminal 268 AA region, but not when it was further shortened to the N-terminal 180 AA. Conversely, deletion of as little as 50 AA from the N-terminus abolished the transcriptional activation of TIP. These data mapped the transcriptional activator activity to the N-terminal 268 AA portion. In contrast, the CP-binding domain of TIP appeared to map exclusively to the C-terminal 100 AA residues. This
is based on the observation that all of the C-terminal deletions failed to interact with the CP and the fact that this region alone was sufficient for CP interaction.

**TIP binds to DNA nonspecifically**

The fact that TIP activated transcription of the reporter gene in yeast cells prompted the speculation that TIP was likely a transcription factor. Indeed, several other NAC proteins have been shown to be transcriptional activators (Duval et al., 2002, Xie et al., 2000). Arabidopsis proteins NAC1 and AtNAM, like other known transcription factors, were also shown to bind DNA. Here we report the results of in vitro DNA-protein binding experiments in an effort to determine the nature of any TIP DNA-binding properties. For these experiments, we used the N-terminal 268 AA portion of TIP (N-268) that we showed in the previous section was responsible for the transcriptional activation activity. Moreover, the DNA-binding activity of both NAC1 and AtNAM has been previously mapped to the N-terminal conserved NAC domain (Duval et al., 2002, Xie et al., 2000). The purified His-tagged N-268 was mixed with a PCR-generated, ^32P-labelled DNA fragment of 68 bp that contained 20 random nucleotides flanked by restriction enzyme digestion sites (BamHI and HindIII). The results of a typical binding experiment are shown in Figure 3A. Note that TIP was able to bind to and retard the mobility of DNA fragments to discreetly shifted bands in lanes 1 and 2 (marked by arrows, also in lane 6), which likely represents homodimerization of TIP. To determine if this binding was specific for a sequence motif, the individual shifted bands were excised, recovered, amplified by PCR, and subjected to a second cycle of binding with the N-268 peptide. This process of binding assays was repeated for several cycles in an effort to enrich for potential DNA fragments with specific TIP-binding sequences. Lanes 4–6 in Figure 3A show the result of a third cycle of binding assays. A comparison of the results of the first (lane 2) and third cycle (lane 6) failed to reveal any significant quantitative difference in the amount of bound DNA. This indicated that the process did not lead to enrichment of DNA fragments that bound to TIP preferentially. We conclude from these results that TIP does not bind DNA in a sequence-specific manner. This conclusion was further supported in the assays using poly(dI:dC) as a competitor in the third round of binding experiments (Figure 3B). It is evident that increasing the amount of poly(dI:dC) reduced the binding of TIP to the labeled DNA proportionally (Figure 3B, lanes 2–6). Although these experiments establish that the truncated form of TIP binds to DNA nonspecifically, we cannot be sure from these experiments if the level of binding was significant. Moreover, we cannot completely rule out the possibility that full-length TIP, which was not used in these assays, might bind DNA more specifically.

**TIP localizes to nuclei in Nicotiana benthamiana leaves**

An additional characteristic of transcription factors is that they localize to the nucleus of the cell. Having shown that TIP activates transcription in yeast cells, we next wanted to test if TIP would localize to the nuclei of plant cells.
Initial examination of the TIP sequence identified a region that could potentially form two overlapping putative nuclear localization signals (NLSs) of a bipartite nature (Figure 4A), which is typical for NLSs of plant origin (Varagona et al., 1992). To experimentally demonstrate the nuclear localization of TIP, we utilized agro-infiltration to deliver green fluorescence protein (GFP)-tagged TIP (GFP-Ala$_{10}$-TIP, see Experimental procedures for details about the constructs used) into the cells of N. benthamiana plants. The infiltrated leaves were collected 2 days after infiltration and directly observed by confocal microscopy (Figure 4B). The distribution of green fluorescence throughout the cytoplasm was as expected for the control GFP-Ala$_{10}$ protein (panel 1). As anticipated, the GFP-Ala$_{10}$-TIP fusion protein primarily localized to the nuclei of cells (panel 2). These results were also confirmed by fluorescent microscopy (Figure 4C, top panels). We conclude from these results that TIP localizes to the cell nucleus, further supporting the notion that TIP is a transcription factor.

**Figure 4.** The cellular localization of TIPGFP fusion proteins transiently expressed in N. benthamiana leaves in the presence and absence of TCV CP and mutant CP. (A) Sequence of a portion of TIP showing the two overlapping putative nuclear localization signals (NLS 1 and 2). (B) Confocal microscopy showing the cellular distribution of GFP alone (panel 1), GFP-TIP fusion alone (panel 2), and co-expression of GFP + TCV CP (panel 3), GFP-TIP fusion + TCV CP (panel 4), GFP-TIP fusion + TCV CP R6A (panel 5), and GFP-TIP fusion + TBSV CP (panel 6). (C) Fluorescent microscopy showing cell nuclei stained with DAPI after transient expression of the GFP-TIP fusion protein alone (top panels) and in the presence of TCV CP (middle panels) and the TCV CP R6A mutant (bottom panels). The panels on the right show the merged image of the DAPI-stained nuclei and the GFP-stained proteins. Note the absence of colocalization of the two signals in the middle panel in the presence of the wild-type TCV CP.
**Discussion**

We have previously described the identification of an *A. thaliana* protein TIP that interacts with TCV CP through yeast two-hybrid screening of an *A. thaliana* cDNA library. We further noted that this CP–TIP interaction correlated with the resistance response conferred by *A. thaliana* ecotype Dijon-17 to TCV. We also showed that TIP activates the transcription of the reporter genes from a GaL4 promoter in the absence of GaL4 transcriptional activator, hinting that TIP might also be a transcriptional activator (Ren et al., 2000). In this current report, we have further characterized the functions of TIP and provide evidence for a possible role of TCV CP in mediating the viral–host interaction. We have now verified the physical CP–TIP interaction in in vitro binding assays. Mutant CPs that failed to interact with TIP in yeast cells were shown to bind TIP more weakly than either the wild-type CP or the mutant that retained interaction with TIP in yeast cells. In addition, we have shown that TIP tagged with GFP (GFP–Ala10–TIP) localized to the nuclei of plant cells and that the nuclear localization of TIP was disrupted by TCV CP co-expression. We also mapped the CP-interacting domain of TIP to the C-terminal 100 AA region of this 451 AA protein. The transcriptional activation function, on the other hand, was mapped to the N-terminal 268 AA region, which is the region conserved among all members of NAC protein family (Aida et al., 1997). This N-terminal 268 AA region was further shown to bind to DNA, albeit nonspecifically. Together, these results suggest that TIP is a transcription factor that functions in the defense response of *A. thaliana* to virus invasion. In our model (see later), TCV counters this defense through specific interaction with TIP.

We were initially a little surprised by the result that the TCV CP mutants unable to interact with TIP in yeast cells were capable of binding to TIP in vitro. However, careful quantitative analysis revealed that the binding ability of mutant CPs with TIP was significantly weaker than the binding of wild-type TCV CP. It is important to note that a strict correlation exists between interaction in yeast cells and binding efficiency in vitro. Mutant CPs incapable of TIP interaction in yeast invariably showed weaker TIP-binding in vitro, whereas the single mutant CP (G14A) capable of TIP interaction in yeast bound to TIP in vitro as strongly as wild-type CP. We conclude that the 5-fold weaker binding ability measured in vitro between TIP and the mutant CPs precluded their ability to function in vivo. This is also supported by the inability of mutant CP (R6A) to prevent the nuclear localization of GFP-tagged TIP in plant cells.

That TIP is a transcription factor was first inferred from previous reports that members of NAC protein family, to which TIP belongs, were found to be transcription factors (NAC1, Xie et al., 2000) or be able to activate transcription (ATAF1 and ATAF2, Souer et al., 1996). Additional NAC proteins were found to bind the CaMV 35S promoter DNA (Duval et al., 2002, Xie et al., 1999). In addition, all NAC proteins possess nuclear localization signals. Evidence supporting TIP as a transcription factor now includes (1) TIP activates transcription in yeast cells; (2) TIP localizes to the nuclei of plant cells; and (3) TIP binds to DNA. The observation that TIP binds to DNA nonspecifically suggests that TIP might activate transcription indirectly through a protein complex containing other factor(s) conferring the DNA-binding specificity. However, since full-length TIP was not used in the DNA-binding experiments, caution must be exercised in interpreting these results.

Evidence for TIP as a component in the host defense mechanism is still circumstantial and derived from the fact that the CP–TIP interaction correlates with the activation of the resistance response in *A. thaliana* ecotype Dijon-17. This ecotype harbors the R gene *HRT*, a typical *R* gene with nucleotide-binding sites (NBS) and leucine-rich repeats (LRR) (Cooley et al., 2000, Dangl and Jones, 2001).
However, the fact that TIP is present in both TCV-susceptible and -resistant *A. thaliana* ecotypes, most likely at similar expression levels (Ren et al., 2000), suggests that the HRT protein is not needed for the CP–TIP interaction. This is further supported by results showing that, in cells of an unrelated plant species, TCV CP interacts with TIP and prevents its nuclear localization. Assuming TIP is indeed a transcription factor, its nuclear localization would be essential for its function. Therefore, it can be inferred that the viral CP has been selected for the ability to bind TIP and prevent it from functioning properly because TIP must somehow interfere with viral invasiveness. Conversely, the proper functioning of TIP might have an adverse effect on TCV multiplication. In either case, we suggest TIP might be considered a component in the host basal defense pathways, as defined in the “Guard hypothesis”. This hypothesis was first proposed to explain the mechanism of gene-for-gene resistance in plants mediated by NBS–LRR class of R genes like HRT (Cooley et al., 2000).

Despite the fact that the CP–TIP interaction does not require HRT, the correlation between CP–TIP interaction and the activation of resistance clearly shows that HRT-mediated resistance requires positive CP–TIP interaction. The intricate inter-relation between TCV CP, TIP, and HRT revealed in our work prompts us to propose a model to explain their mutual interaction (Figure 5). In this model, TIP is proposed to be a transcription factor regulating some aspect of the basal anti-viral defense machinery in *A. thaliana*. To ensure successful multiplication, viruses like TCV would have evolved mechanisms to interfere with proper functioning of TIP. In the case of TCV, CP has evolved the ability to block the nuclear localization of TIP. In response to the detrimental impact on plant normal development brought about by rigorous TCV replication, some ecotypes of *A. thaliana* (e.g., Dijon-17) in turn evolved a counter defense using the HRT gene, whose protein product guards TIP. Changes in TIP (for example, abnormal cellular localization, or complex formation with foreign proteins) are quickly detected by HRT, which then triggers the resistance cascade leading to cell death that contains the TCV invasion. This model is consistent with the “Guard hypothesis” (Dangl and Jones, 2001, Schneider, 2002, van der Biezen and Jones, 1998), which is currently the most prevalent theory for explaining the NBS–LRR class R gene function. In this hypothesis, the pathogen effectors, products of genes that were previously defined as *avr* genes, would act instead as virulence factors to attack key components in the host basal resistance machinery (also termed ‘guardee’). The function of the typical NBS–LRR resistance proteins is then to guard these key components of basal resistance machinery. Changes in the guardee molecules caused by effectors are monitored and sensed by guard molecules (R protein) that then activate the resistance pathway. Elegant examples include the RPM1 and RPS2 R genes of *A. thaliana*, which mediate resistance responses to invasions of different species of *P. syringae* (Axtell and Staskawicz, 2003, Mackey et al., 2002, Mackey et al., 2003). It is well known that the RPM1 gene confers resistance to *P. syringae* harboring *AvrB* or *AvrRpm1* genes, and RPS2 gene confers resistance to *P. syringae* with *AvrRpt2* gene. Recently, it has been discovered that one cellular factor, RIN4, which is most likely an activator of the basal plant defense, is involved in the race-specific resistance responses mediated by both RPM1 and RPS2. The direct interaction of RIN4 with AvrB or AvrRpm1 leads to the phosphorylation of RIN4, which is detected by RPM1, whereas interaction between RIN4 and AvrRpt2 leads to degradation of RIN4, which triggers RPS-mediated resistance. To date, TIP remains the only known example of a host factor that is involved in *A. thaliana* resistance to a viral pathogen and for which both the viral effector and host resistance protein are known.

**Experimental procedures**

**In vitro protein–protein binding assay**

In vitro protein binding assays were performed using glutathione-S-transferase (GST) fusion protein of TIP (GST-TIP) and in vitro-translated CP derivatives. To produce GST-TIP, the TIP cDNA was cloned into vector pGEX-4T-1 to make the construct pGEX-4T-1-TIP, which was

![Figure 5](image-url)
transformed into *Escherichia coli* strain BL21. The GST-TIP fusion protein was then purified from BL21 using Glutathione Sepharose 4B matrix, following the manufacturer’s specifications (Amersham Pharmacia Biotech, Uppsala, Sweden), except that 50 μM instead of 500 μM of isopropyl-β-D-thiogalactoside (IPTG) was used to induce the expression of the GST fusion protein.

The radioactively labeled proteins (TCV CP, the RNA-binding domain [R domain] of CP and its mutants) were produced by cloning the respective cDNAs into pBluescript II SK between EcoRI and PstI sites, followed by linearization of the derived plasmids with XbaI, and coupled transcription and translation in the presence of [35S]methionine using the TNT-coupled wheat germ extract system (Promega, Wisconsin, MI). Translation products were analyzed by 10% SDS-polyacrylamide gel followed by autoradiography.

The in vitro protein binding experiments were carried out as described by Choi *et al.* (2000). Twenty microliters of Sepharose beads with GST-TIP attached was incubated with 25 μl of in vitro translation mixture in a total volume of 300 μl of binding buffer (50 mM Tris–HCl, pH 7.6, 200 mM NaCl, 0.1% Triton X-100) at 4 °C for 3 h with gentle rocking. Beads were then collected, washed, and resuspended in 20 μl of 2× loading buffer (100 mM Tris–HCl, pH 6.8, 200 mM DTT, 4% SDS, 0.2% bromophenol blue, 20% glycerol) for SDS-PAGE. The gels were then dried and exposed to X-ray film. For quantitative analysis, the experiments were repeated for selected CP derivatives and the resulting films were scanned using a densitometer (Amersham Biosciences).

**Yeast two-hybrid analysis**

The MATCHMAKER Two-Hybrid System 2 (Clontech, Palo Alto, CA) was used for the yeast two-hybrid experiments. The activation domain (AD) vector used was pGAD10 and the DNA-binding domain (BD) vector was pAS2-1. The yeast strain was Y190. The experiments were carried out following the manufacturer’s specifications.

**In vitro binding of TIP and DNA**

For the in vitro DNA-binding experiments, the N-terminal 268 AA portion of TIP was produced as a TIP-His fusion protein in *E. coli* using the pET-28a expression system (Novagen, Madison, WI) and purified with the His-Bind Quick Column according to manufacturer’s specifications (Novagen). The double-stranded DNA probe was generated by annealing primer C (5’-GCC GAC GTA AGC TTC GGA AG-3’, underlined is the recognition site for *HindIII*) to a 10-fold molar excess of the oligonucleotide A (5’-GTC TGT CTG CAT CCC AGG TGA GTA N20 ACG TCT TCC GAA GCT TAC GTC GCG-3’, underlined are recognition sites for *BamHI* and *HindIII*, respectively), which contained 20 random nucleotides in the middle (modified after Martinez-Garcia *et al.*, 2000), followed by elongation with the Klenow fragment of *E. coli* DNA polymerase. The double-stranded products were separated from single-stranded oligonucleotide on an 8% polyacrylamide gel and purified. They were then radioactively (32P) end-labeled by T4 kinase.

The DNA binding assays were carried out using a procedure modified after Blackwell and Weintraub (1990). The reactions were performed at room temperature for 30 min in a buffer (Molloy, 2000) containing 4% Glyceral, 1 mM MgCl₂, 0.5 mM EDTA, 0.5 mM DTT, 50 mM NaCl, and 10 mM Tris–HCl, pH 7.5 with different amounts of TIP–His, and random dsDNA fragments. The reactions were then loaded on 6% polyacrylamide gels that were prerun in 0.5× TBE buffer for 10 min at 350 V. The gel was then run at 4 °C, 350 V until the bromophenol blue dye was just off the gel (less than 20 min). The band containing the DNA fragments of slower mobility was excised and incubated at 37 °C for 3 h in 0.5 ml extraction buffer (0.5 M ammonium acetate, 10 mM MgCl₂, 1 mM EDTA, and 0.1% SDS). The recovered DNA fragments were precipitated with ethanol and amplified by PCR with primers B (GTCTGTCTGGATCCAGGGTG) and primer C.

**Nuclear localization of TIP**

Transient expression of proteins in plant leaf cells was accomplished with the Agrobacterium infiltration procedure (Qu *et al.*, 2003). Expression cassettes containing cDNAs of individual or fusion proteins sandwiched by Cauliflower mosaic virus 35S promoter and terminator sequences were cloned into the binary vector pZP212 and transformed into Agrobacterium strain C58C1 (Qu *et al.*, 2003). The constructs used in this experiment were PZP–GFP–Ala₁₀ PZP–GFP–Ala₁₀–TIP, PZP–TCVCP, PZP–TCVCP–R6A, and PZP–TBSVCP. PZP–GFP–Ala₁₀–TIP is designed to express the green fluorescence protein (GFP)–TIP fusion protein in plant cells. It included 10 alanine residues (Ala₁₀) inserted between GFP and TIP to facilitate correct protein folding. Accordingly, the control construct PZP–GFP–Ala₁₀ expressing the modified version of GFP also had 10 alanine residues at its C-terminus. PZP–TCVCP and PZP–TCVCP–R6A would enable the expression of TCV CP and its mutant R6A. PZP–TBSVCP is an additional control that expresses the coat protein of Tomato bushy stunt virus (TBSV). Agrobacterium suspensions carrying the various binary constructs were pelleted and resuspended in a solution containing 10 mM morpholinepropanesulfonic acid (pH 5.5), 10 mM MgCl₂, and 100 μM acetosyringone to an optical density of 1.0 at 600 nm. In co-inoculations, equal volumes of each suspension were mixed prior to infiltration. Three-week-old *N. benthamiana* were infiltrated on the first two true leaves with a 3-ml, needleless syringe. The infiltrated plants were kept in growth chambers for a 12-h day length at a daytime temperature of 24 °C and a nighttime temperature of 22 °C.
Microscopy

Agro-infiltrated N. benthamiana leaves were harvested at 2 days post-infiltration (dpi), mounted in water, and viewed by confocal fluorescence microscopy using a Bio-Rad MRC 1024ES laser scanning confocal microscope system. GFP fluorescence was visualized by using dual excitation emission (Ex: 488/640 nm; Em: 522/680 nm). The plant cell nuclei were stained by direct infiltration of N. benthamiana leaves with 1 μg/ml 4',6-diamidino-2-phenylindole (DAPI). DAPI-stained leaves were mounted in water and viewed with an Olympus AX 70 fluorescence microscope.

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