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A nuclear fraction of turnip crinkle virus capsid protein is important for elicitation of the host resistance response

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Abstract

The N-terminal 25 amino acids (AAs) of turnip crinkle virus (TCV) capsid protein (CP) are recognized by the resistance protein HRT to trigger a hypersensitive response (HR) and systemic resistance to TCV infection. This same region of TCV CP also contains a motif that interacts with the transcription factor TIP, as well as a nuclear localization signal (NLS). However, it is not yet known whether nuclear localization of TCV CP is needed for the induction of HRT-mediated HR and resistance. Here we present new evidence suggesting a tight correlation between nuclear inclusions formed by CP and the manifestation of HR. We show that a fraction of TCV CP localized to cell nuclei to form discrete inclusion-like structures, and a mutated CP (R6A) known to abolish HR failed to form nuclear inclusions. Notably, TIP-CP interaction augments the inclusion-forming activity of CP by tethering inclusions to the nuclear membrane. This TIP-mediated augmentation is also critical for HR resistance, as another CP mutant (R8A) known to elicit a less restrictive HR, though still self-associated into nuclear inclusions, failed to direct inclusions to the nuclear membrane due to its inability to interact with TIP. Finally, exclusion of CP from cell nuclei abolished induction of HR. Together, these results uncovered a strong correlation between nuclear localization and nuclear inclusion formation by TCV CP and induction of HR, and suggest that CP nuclear inclusions could be the key trigger of the HRT-dependent, yet TIP-reinforced, resistance to TCV.

Keywords

Turnip crinkle virus; carmovirus; plant virus; antiviral defense; nuclear localization; resistance gene; GFP

Introduction

Turnip crinkle virus (TCV) is a member of the family *Tombusviridae*, genus *Carmovirus*. TCV contains a positive sense, single-stranded RNA genome of 4,054 nucleotides

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encapsidated in an icosahedral virion consisting of 180 capsid protein (CP) subunits (Hogle et al. 1986, Sorger et al. 1986). In addition to serving a structural role, CP also participates in many aspects of TCV-host plant interactions including virus movement in various hosts (Cao et al., 2010; Cohen et al., 2000; Li et al., 1998; Hacker et al., 1992), suppression of RNA silencing (Qu et al., 2003; Thomas et al., 2003), and elicitation of TCV-targeting hypersensitive response (HR) and resistance in the Arabidopsis ecotype Di-17 encoding HRT, a nucleotide-binding leucine-rich repeat (NB-LRR) resistance protein (Cooley et al., 2000; Kachroo et al., 2000; Dempsey et al., 1997). Zhu and colleagues (2013) reported that TCV CP elicits HRT-mediated resistance by modulating an interaction between double-stranded RNA binding protein 4 (DRB4) and HRT. DRB4 was shown previously to participate in RNA silencing-mediated defense against TCV (Qu et al., 2008). Therefore, the demonstration of a direct DRB4-HRT interaction provided the first evidence for a possible link between *HRT*-mediated resistance to TCV and the RNA silencing-based antiviral defense.

We reported earlier that TCV CP physically interacts with TIP, an Arabidopsis NAC transcription factor, and that transiently expressed TCV CP disrupted nuclear localization of TIP (Ren et al., 2000; 2005). We speculated then that HRT might guard TIP against TCV invasion by sensing a TIP fraction retained in cytoplasm by TCV CP. However, a subsequent study discounted a direct role of TIP in HRT-mediated TCV resistance by showing that TIP was not required for HR, but rather was involved in basal defense response in Arabidopsis against both TCV and cucumber mosaic virus (Jeong et al., 2008). Our lab subsequently confirmed a primary role of TIP in the TCV-targeting basal resistance (Donze et al., 2014).

TCV CP possesses several functional domains that serve distinct roles in capsid assembly (Hogle et al., 1986). The amino-terminal 52 amino acid (AA) residues constitute the R domain that binds genomic RNA. We previously showed that R domain was also responsible for eliciting HRT-mediated resistance in Arabidopsis, as certain single AA substitutions in a defined region of R domain caused CP to lose the ability to trigger HRT-mediated defense in resistant Di-17 plants. Importantly, these same CP mutants also displayed reduced affinity with TIP (Ren et al., 2000; 2005). Donze and colleagues (2014) went on to show that one such mutant, R6A, was less invasive than wildtype (WT) TCV in the susceptible Col-0 ecotype of Arabidopsis, presumably due to inability to down-regulate gene expression associated with onset of the basal resistance response. These observations prompted the hypothesis that TIP functions as a negative regulator of basal resistance through interaction with the R domain of TCV CP.

We showed in a recent study (Kang et al., 2015) that transient expression of the R domain of TCV CP in the presence of HRT was sufficient to induce HR in *Nicotiana benthamiana*, and that mutant R domains with single AA mutations displayed varying HR-eliciting activities that correlated with the resistance responses triggered by the corresponding mutant viruses in Arabidopsis plants. These results demonstrated that the TCV R domain alone is sufficient for the elicitation of HRT-dependent HR and resistance. Considering the fact that this same domain is also responsible for TIP binding, the dispensability of TIP in HR and resistance

elicitation led us to speculate that certain other features of R domain, though overlapping with TIP-binding region, might act independently to induce HR and systemic resistance.

We have now resolved this conundrum with the results reported here. First, we have confirmed the presence of a nuclear localization signal (NLS) in CP R domain and showed that it overlaps the TIP binding region (Fig. 1A). We further demonstrated that a fraction of TCV CP translocates to cell nuclei where it forms punctate inclusions. Interestingly, the non-HR inducing R6A mutant of CP also translocated to cell nuclei but failed to associate into inclusion-like structures. In addition, we found that CP variants capable of TIP-binding (WT CP and G14A CP) accumulated at the periphery of nuclei in the presence of TIP, while localization of CP mutants unable to bind TIP (R6A CP and R8A CP) was not changed by TIP expression. These results suggest that the nuclear localization of TCV CP is a previously unidentified property of CP needed for HR induction.

2. Materials and Methods

2.1. Plasmid construction

Constructs designed to express CP and its mutants tagged with GFP at the amino terminus (GFP-CP)—The cDNA fragment of GFP was amplified with primers that incorporate an *Apa*I recognition sequence at the end and an *AsiS*I recognition sequence at the 3′ end. The template for GFP was the cDNA of mGFP4 (Haseloff et al., 1997), which originated from the genomic DNA of GFP transgenic 16c plants. The innate stop codon of the GFP ORF was eliminated to permit continuous expression of the downstream CP. CP WT and other CP mutants were amplified by the same set of primers with an *AsiS*I recognition sequence on 5′ end and an *Xba*I recognition sequence on 3′ end (Fig. 1B). The amplified CP fragments were ligated into the pRTL4i vector.The GFP cDNA was subsequently fused in-frame with the CP cDNAs.

Constructs designed to express CP and its mutants tagged with GFP at the carboxyl terminus (CP-GFP)—The cDNA fragment of GFP was amplified with primers that incorporate a *Not*I recognition sequence on the 5′ end and an *Xba*I recognition sequence on the 3′ end. Both CP WT and other CP mutants were amplified by the same primers that incorporate an *AsiS*I recognition sequence on the 5′ end and a *Not*I recognition sequence on the 3′ end. The CP stop codon of CP was eliminated to permit contiguous expression of downstream GFP (Fig. 1B). These CP fragments were fused in-frame with GFP cDNA, and ligated into the pRTL4i vector.

Constructs designed to express GFP-CP-NES and GFP-R6A-NES—The CP region of GFP-CP construct was replaced with CP from CP-GFP to obtain a CP ORF lacking the stop codon. Two complementary oligomers of the sequence of the nuclear export signal (NES) from protein kinase inhibitor (Gadal et al., 2001) with a *Not*I recognition sequence on the 5′ end and an *Xba*I recognition sequence on the 3′ end were annealed and ligated at the 3’ end of GFP-CP (Fig. 1B). The whole expression cassette was cut with *Apa*I and *Xba*I and then inserted into pRTL4i.

Other constructs. HRT—HRT was previously cloned in pSK plasmid with a myc (N-EQKLISEEDL-C) tag attached on its 3′ end. Cloned pSK plasmid was cut by *Spe*I and a blunt end was generated by T4 DNA Polymerase (NEB) for subsequent cloning into pRTL4i vector (Kang et al., 2015). All constructs in pRTL4i plasmid were digested with *Sbf*I and subsequently cloned into pSW4i vector, which was derived from the pZP212 plasmid (Hajdukiewicz et al., 1994). Final constructs were verified by PCR and sequencing. **RFP-*At*Fib**. A plasmid expressing RFP tagged *A. thaliana* fibrillarin was obtained from Dr. Taliansky's group at the Scottish Crop Research Institute (Kim et al., 2007).

2.2. Agrobacterium infiltration

All pSW4i clones were introduced into the *Agrobacterium tumefaciens* strain C58C1 by electroporation. Transformed cells were plated on LB+4 (Streptomycin, Rifampicin, Gentamicin and Carbenicillin) agar media and incubated for 2 days at 30°C. Colonies were re-streaked on a new LB+4 agar media and grown in LB+4. Agrobacterium cultures were grown overnight and centrifuged. Cell pellets were re-suspended with MES solution containing 10 mM 4-morpholinepropanesulfonic acid (pH 5.5), 10 mM $MgCl_2$, and 100 uM acetosyringone to an optical density at 600 nm of 0.6. Cell suspensions were kept at room temperature for 2 hours prior to infiltration. For co-infiltration, equal volumes of each Agrobacterium suspension were mixed. Three-week-old *N. benthamiana* plants were infiltrated on the first two true leaves with a needless syringe. Infiltrated plants were covered with a transparent plastic lid and placed in a growth chamber programmed with a 12 hour day and 12 hour night cycle at 22 °C for up to 7 days. The plastic lid was removed after one night. Unlike other GFP-tagged constructs, which were grown on LB+4 media, RFP-*At*Fib transformed cells were grown on LB+3 (Rifampicin, Gentamicin and Kanamycin) media.

2.3. Microscopy

Subcellular localization of the fluorescent protein tagged CPs was examined using confocal laser-scanning fluorescence microscope (CLSFM). Infiltrated *N. benthamiana* leaves were cut and mounted with water on glass slides. For each infiltration treatment, in excess of 18 cells (6 cells per prepared leaf sample, 3 samples per treatment) were observed with consistent results, and the same treatment was repeated a minimum of three times. Samples were covered with cover slips and viewed under the BIORAD MRC 1024ES confocal laser-scanning microscope. The fluorescence was measured simultaneously with 2-line laser excitation mode (488 nm and 560 nm) and the emission filter set (522 nm and 598 nm) using BioRad LaserSharp imaging program.

3. Results

3.1. A fraction of transiently expressed, GFP-tagged TCV CP localizes to cell nuclei to form inclusion-like structures

TCV CP accumulates primarily in the cytoplasm of TCV-infected cells where virion assembly takes place (Blake et al., 2007). Nevertheless, it was predicted to contain a putative nuclear localization signal (NLS) (Kang, 2012). This putative NLS encompasses AA #6 to #24 (RVRKFASDGAQWAIKWQKK; Fig. 1A), and thus overlaps the previously identified TIP-binding region (Ren et al., 2000; Fig. 1A). To determine whether TCV CP

indeed localizes to cell nuclei, we fused the GFP ORF to either the amino- or carboxyl-terminus of CP to produce constructs GFP-CP and CP-GFP, respectively (Fig. 1B).

These constructs were introduced into cells of *N. benthamiana* plants by agro-infiltration to express the corresponding fusion proteins. At two days after infiltration (DAI), leaves were collected and examined using confocal microscopy (Fig. 2). For both GFP-CP and CP-GFP constructs, intense and diffused GFP fluorescence were observed in the cytoplasm of treated cells (Fig. 2B, left panel; and data not shown). However, examination of nuclei of treated cells showed that both fusion proteins were also detectable in nuclei as discrete inclusions (Fig. 2B, right two panels). Note that these inclusions were consistently observed in multiple experiments, and were not present in cells expressing free GFP (Fig. 2A), indicating that they are CP-induced. Additionally, GFP fluorescence was also observed in cell nucleoli in the diffused form (Fig. 2B).

To determine whether nuclear inclusions depended on the NLS described above, we further assessed whether the CP of R6A mutant, in which the arginine residue at AA #6 was mutated to an alanine, could induce similar inclusions. As shown in Fig. 2C, cells expressing both GFP-R6A and R6A-GFP showed a diffused distribution that was similar to that of free GFP (Fig. 2A). Interestingly, GFP fluorescence was still detected in cell nuclei, and sometimes in nucleoli as well (Fig. 2C). These results suggested that a fraction of R6A CP could still localize to nuclei. However, the ability of TCV CP to form nuclear inclusion bodies was lost as a result of the R6A mutation. Since the R6A mutant was shown to have lost the ability to induce HRT-dependent HR in our earlier studies (Kang et al., 2015), these results imply that formation of inclusion-like bodies might be needed for HR induction by WT CP.

3.2. Formation of nuclear inclusions by TCV CP correlates with induction of HRT-dependent HR

Having observed that WT CP, but not the R6A mutant, formed nuclear inclusion bodies of considerable size that sometimes equaled the size of nucleoli, we next determined if these inclusion bodies co-localized with nucleoli of treated cells. Since the plant protein fibrillarin (Fib) is known to be abundantly present in the nucleoli (Kim et al., 2007), we co-expressed the RFP-tagged Fib of Arabidopsis (RFP-*At*Fib) with the GFP-CP and GFP-R6A constructs. As shown in Fig. 3, second row, nucleoli were easily visible as intensely red spheres (n in Fig. 3), which did not overlap with inclusion-like structures formed by GFP-CP (I in Fig. 3). To further assess if the formation of inclusion-like structures correlated with HR induction, we evaluated the localization of two additional single AA mutants of CP that retained the ability to induce HRT-dependent HR (R8A and G14A; Fig. 1A; Kang et al., 2015). As shown in Fig. 3 (right two rows of panels), both of these mutants retained the ability to induce inclusion-like structures in the nuclei of treated cells. These results support the assertion that formation of inclusion-like structures is critical for induction of HRT-dependent HR by TCV CP.

3.3. Co-expression of TIP translocates TCV CP inclusions to nuclear peripheries

Results from previous sections strongly suggest that the R domain of TCV CP, especially the first 25 AA residues that contain the overlapping motifs responsible for TIP-binding and nuclear localization (Fig. 1A), are critical for aggregation into observed nuclear inclusions. In an attempt to uncouple TIP-binding and inclusion formation, we next tested whether co-expression of TIP affects the occurrence and the shape of nuclear inclusions. The R6A, R8A, and G14A mutants were again included because our previous studies showed that these mutants differ in binding affinity to TIP: R6A and R8A, but not G14A, caused CP to lose interaction with TIP (Ren et al., 2000; 2005).

As expected, TIP co-expression caused little change in GFP-R6A distributions (Fig. 4, 2nd column panels). Interestingly, co-expression of TIP led to an increase of the number and intensity of nuclear inclusions formed by WT CP and G14A, but not of those formed by R8A (Fig. 4). More intriguingly, TIP co-expression caused the GFP-CP and GFP-G14A inclusions to coalesce at nuclear membranes, at times leading to the enclosure of the nucleus by intensely fluorescent inclusion structures (Fig. 4, far left and far right columns). These results suggest that TIP may be localized to the nuclear membrane and thereby anchor TCV CP inclusions. Furthermore, the fact that co-expression of TIP with the non-TIP-interacting GFP-R8A failed to cause similar exacerbation of inclusion aggregation or coalescence toward the nuclear membrane, strongly suggests that while aggregation of nuclear inclusions is an inherent property of TCV CP, its interaction with TIP augments this property, which could in turn enhance the HRT-mediated defense response. These results identified a novel supportive role for TIP in plant defense against TCV.

3.4. Exclusion of TCV CP from the nucleus abolishes HRT-dependent HR

The correlation between ability of TCV CP to induce nuclear inclusion-like structures and to elicit HR suggests that HR manifestation might require nuclear importation of TCV CP. We hence wanted to test whether TCV CP-induced, HRT-dependent HR would be abolished if TCV CP was prevented from entering nuclei. Since single AA substitutions within the NLS did not abolish the nuclear targeting of the mutant CPs (R6A, R8A, or G14A), we decided to use the alternative approach of tagging CP with a nuclear export signal (NES). The NES sequence derived from a yeast protein kinase inhibitor (PKI) (Gadal et al., 2001) was attached to the carboxyl-terminus of both GFP-CP and GFP-R6A to create GFP-CP-NES and GFP-R6A-NES, respectively (Fig. 1B). As illustrated in Fig. 5A, both fusion proteins, but especially GFP-CP-NES, showed visibly less GFP fluorescence in the nuclei than GFP-CP or GFP-R6A. More importantly, GFP-CP-NES abolished formation of nuclear inclusions associated with GFP-CP expression (Fig. 5A, 2nd panel).

One observation made in our previous reports was that CP-TIP interaction could prevent TIP from localizing to cell nuclei (Ren et al., 2000). Now that this observation appears to be contradicted by our more careful assessment with the upgraded confocal microscope, we wanted to assess the opposing possibility of TIP-assisted nuclear import of TCV CP. We hence tested whether GFP-CP-NES, which was kept in the cytoplasm by NES, could be translocated to the nucleus through its interaction with TIP. As shown in Fig. 5B, 2nd panel, co-expression of GFP-CP-NES and TIP failed to restore nuclear localization to GFP-CP-

NES. Therefore, TIP-CP interaction likely occurs only in cell nuclei after TCV CP entry, and this interaction serves to reinforce the HRT-mediated defense response. Consistent with this hypothesis, we found that CP kept in the cytoplasm by NES (CP-NES) lost the ability to induce HRT-dependent HR in *N. benthamiana* leaves (Fig. 5C). Together these results demonstrate that abolishing nuclear localization of TCV CP is enough to disrupt induction of HRT-dependent HR in *N. benthamiana*.

4. Discussion

Previous studies in our lab have implicated the amino-terminally located R domain of TCV CP in elicitation of HRT-mediated resistance to TCV, and interaction of TCV CP with the Arabidopsis transcription factor TIP. The observation that most of the single AA mutations within this domain that disrupted TIP-CP interaction also abolished HRT-mediated TCV resistance in Di-17 Arabidopsis plants initially supported the hypothesis that TIP-CP interaction is the key trigger of HRT resistance. However, this hypothesis was later questioned as mutant plants that do not express TIP were still able to mount HRT-dependent HR to TCV (Jeong et al., 2008). These earlier studies left an important unanswered question: why would the R domain mutations that perturb CP-TIP interactions also affect HRT resistance?

Here we have answered this question with additional cell-biological interrogations of TCV CP and its mutants. We found that the TIP-binding region of TCV CP overlapped with an NLS that translocated a fraction of TCV CP to the nuclei of cells in which TCV CP was expressed. Most notably, we have identified a third property of the TCV CP R domain which is induction of nucleus-specific inclusion bodies of CP. Therefore, mutations within this short domain have the potential to affect one, two, or all three of these properties. Indeed, the three mutants examined in the current study, R6A, R8A, and G14A, displayed varying degrees of perturbation of these three functions. Specifically, R6A had minimal perturbation of nuclear localization but lost the ability to induce nuclear inclusions, or to interact with TIP, whereas R8A was still able to induce nuclear inclusions but no longer interacted with TIP. In contrast, G14A retained all three R domain properties.

We wish to emphasize that formation of inclusion bodies by TCV CP could only occur inside nuclei of the cells, as no CP inclusions were detected in the cytoplasmic fraction of CP. Moreover, excluding CP from entering nuclei completely abolished formation of inclusion bodies. Importantly, a tight correlation between occurrence of nuclear inclusions and induction of HR emerged from our current study as both mutants that retained the ability to associate into nuclear inclusions (R8A and G14A) also retained the ability to induce HRT-dependent HR as reported in our previous study (Kang et al., 2015). Further, disrupting nuclear localization of TCV CP also abolished induction of HRT-dependent HR.

Finally, our study provides the first lead for a clear involvement of TIP in HRT-mediated anti-TCV resistance. We found that nuclear inclusions of WT CP and G14A CP, but not those of R8A CP, were stabilized in the vicinity of the nuclear membrane through interactions with TIP. We reported earlier that the R8A mutation abolished the CP-TIP interaction, and the corresponding R8A mutant of TCV induced a weakened resistance

response in Di-17 plants that permitted systemic expansion of HR in the entire plant (Ren et al., 2000; 2005; Donze et al., 2014; Kang et al., 2015). Based on these results, we propose that TIP-CP association has evolved to bolster the resistance response by stabilizing TCV CP nuclear inclusions in specific nuclear compartments (e.g. nuclear membrane).

It is clear from our results that TCV CP inclusions are present only inside nuclei. Therefore, it remains to be determined what additional nuclear localized host factors are needed to promote aggregation of CP inside cell nuclei. Another related question is whether CP nuclear localization and/or aggregation confer fitness benefit(s) to TCV. The study by Donze and colleagues (2014) showing that R6A induces stronger basal resistance, appears to suggest that nuclear inclusion formation by CP benefits TCV by interferring with the induction of basal resistance. However, we cannot at this point rule out a host-initiated mechanism that compels aggregation of CP in the nuclei. Additional investigations are needed to deepen our understanding of this fascinating model of virus-plant interactions.

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Highlights

- A fraction of TCV CP localizes to cell nuclei and associates into inclusions.
- TIP stabilizes these nuclear inclusions by tethering them to the nuclear membrane.
- CP mutations that perturb HRT-mediated defense compromise nuclear inclusions.
- CP nuclear inclusions appear to be needed for HRT-mediated TCV resistance.

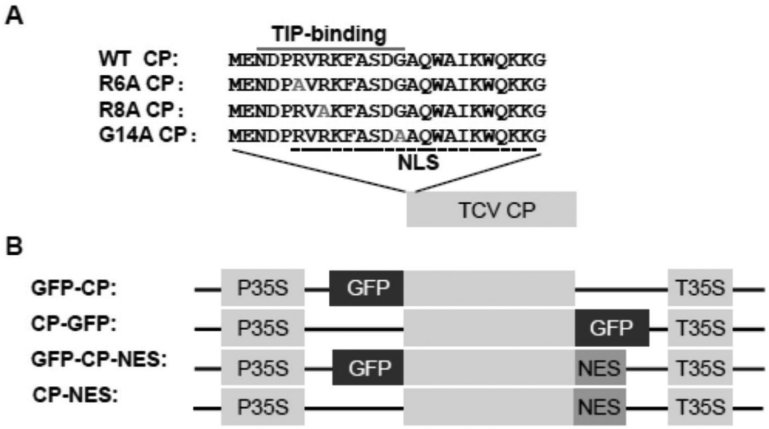

Fig. 1. Putative nuclear localization signal of TCV CP

A. The sequence of the amino-terminal 25 AA of the R domain of TCV CP and mutants used in this study. Residues in the predicted nuclear localization signal (NLS) are shown along with the TIP binding region. Cellular localization prediction byPSORT (http://psort.hgc.jp; Nakai and Kanehisa, 1992) showed a putative NLS (dashed line) overlapping the TIP binding region (solid grey line extended from AA #3 to #13). This putative NLS extended from AA #6 to #24. Putative nuclear localization by PSORT is based on two common nuclear targeting sequences following these rules: i) 4 residue pattern of basic residues (K or R), or of three basic residues (K or R) and H or P, ii) 2 basic residues, 10 residue spacer, and another basic region of at least 3 basic residues out of 5 residues (Robbins et al., 1991). **B.** Schematic diagram of TCV CP constructs. The cDNA of the mGFP4 ORF was fused to the 5' or the 3' of CP ORF. A nuclear exclusion signal (NES) was fused to the 3'end of the CP ORF as described in Materials and Methods.

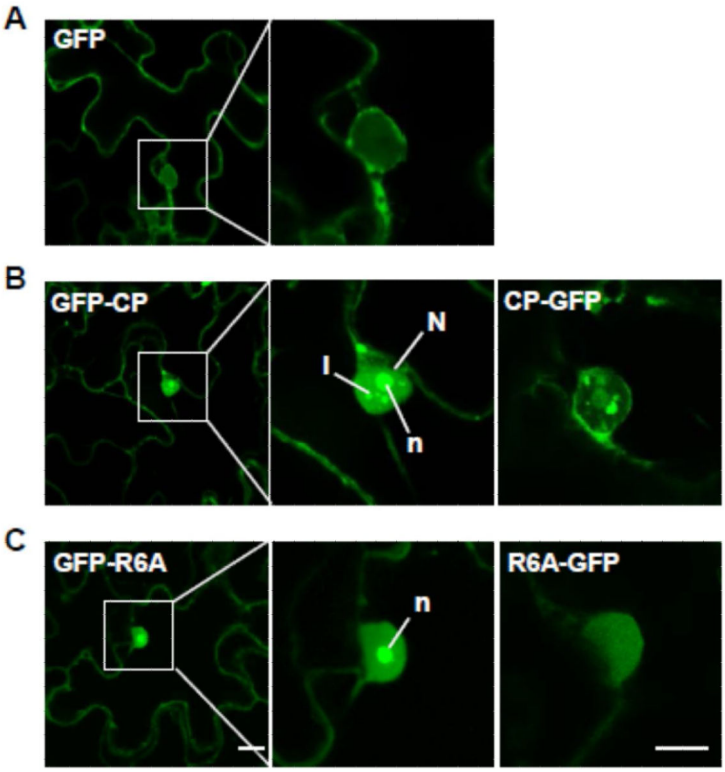

Fig. 2. Subcellular Localization of TCV CP constructs

CLSFM images of GFP-tagged proteins in *N. benthamiana.* At 48 hours post infiltration, infiltrated leaves were cut and mounted on a glass slide. Subcellular structures are indicated (N, Nucleus; n, nucleolus; I, inclusion-like structure; Scale bar, 10 μm). **A.** GFP expressed from a construct containing GFP ORF only is shown as a control. **B.** Vector inserted with WT CP tagged with GFP at both ends (GFP-CP or CP-GFP); Significant accumulation of the GFP-tagged CP was seen in the nucleolus and in more densely fluorescing inclusion-like structures. **C.** Vector inserted with the CP of the R6A tagged with GFP at both ends (GFP-R6A and R6A-GFP. Inclusion-like structures were not observed with these constructs.

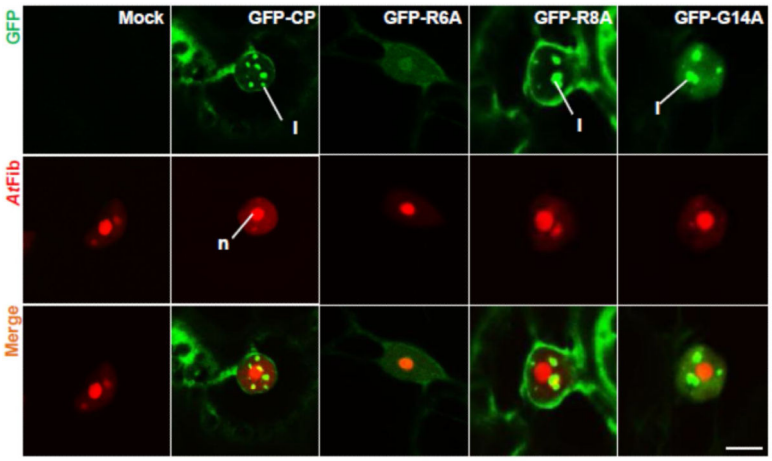

Fig. 3. Inclusion-like structures in the nucleus correlated with the ability of CP to elicit HR

CLSFM images of *N. benthamiana* leaves infiltrated with GFP-tagged CP of WT and three mutants (R6A CP, R8A CP, and G14A CP). Mock plants were treated with RFP-*At*Fib only. (n, nucleolus; I, inclusion-like structure; Scale bar, 10 μm). Each of the HR inducing CPs (WT CP, RSA CP and G 14A CP) showed abundant fluorescence of inclusion-like structures not visible in the non-HR inducing R6A CP infiltrations.

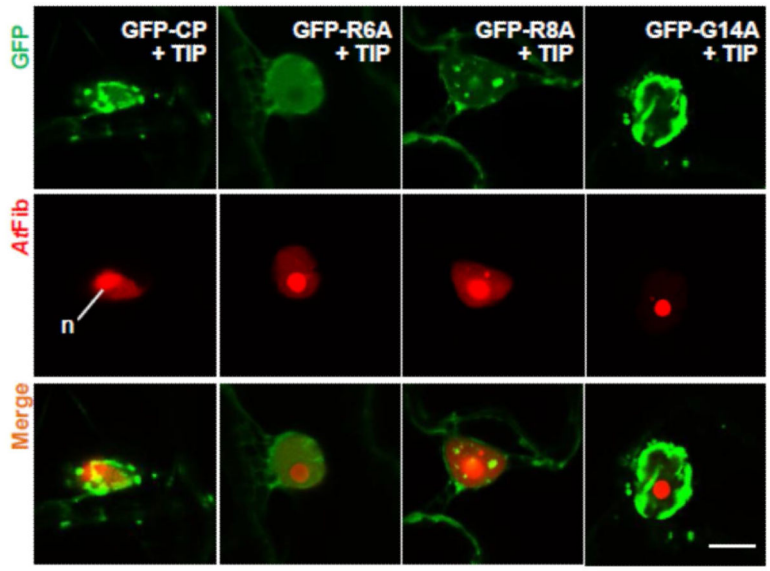

Fig. 4. Subcellular localization of TCV CP in the presence of TIP

The localization of GFP-tagged CPs co-infiltrated with TIP in *N. benthamiana* leaves. At 2 DAI, infiltrated leaves were cut and mounted on glass slides. Note that nuclear localization of the TIP-binding CPs (CP WT and CP G 14A) were more substantially restricted to the periphery of the nucleus compared to the two non-TIP-binding CPs (CP R6A and CP R8A). Scale bar, 10 μm.

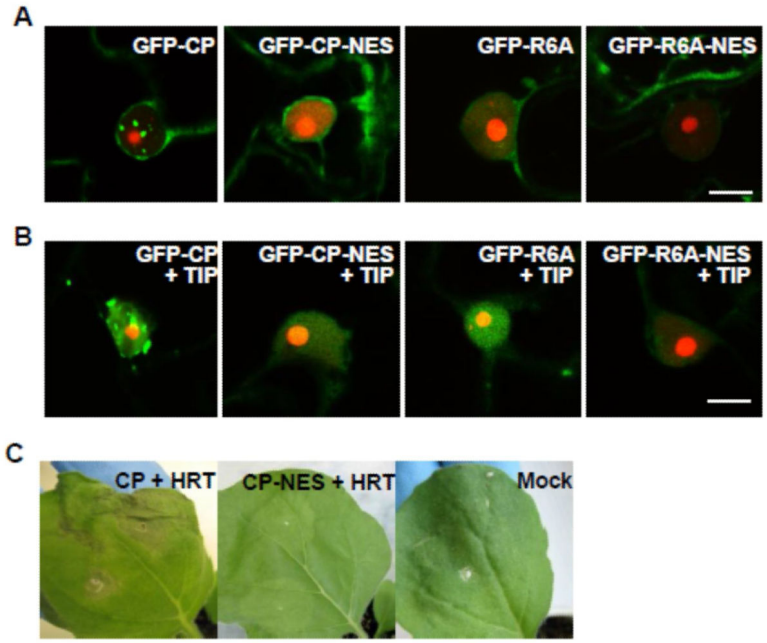

Fig. 5. TCV CPs with an attached nuclear export signal

A. Localization of GFP-tagged CPs with and without a nuclear exclusion signal (NES) sequence inserted at the end of CP ORF. The cells were examined by CLSFM after transient expression in *N. benthamiana* leaves. At 2 DAI, infiltrated leaves were cut and mounted on glass slides. Only the merged channel is shown. **B.** CLSFM images of *N. benthamiana* leaves infiltrated with GFP-labeled CPs with and without NES after co-infiltration with TIP. Scale bar, 10 μm. **C.** HR assays: 4-week-old *N. benthamiana* plants were agro-infiltrated with either WT CP or CP-NES along with HRT to evaluate its ability to induce HR. Leaves were photographed at 4 DAI.