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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.

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1. Introduction

Turnip crinkle virus (TCV) is a member of the family Tombusviridae, genus *Carmovirus*. TCV contains a positive sense, single-stranded RNA genome of 4054 nucleotides 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; Laakso and Heaton, 1993; 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 et al. (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

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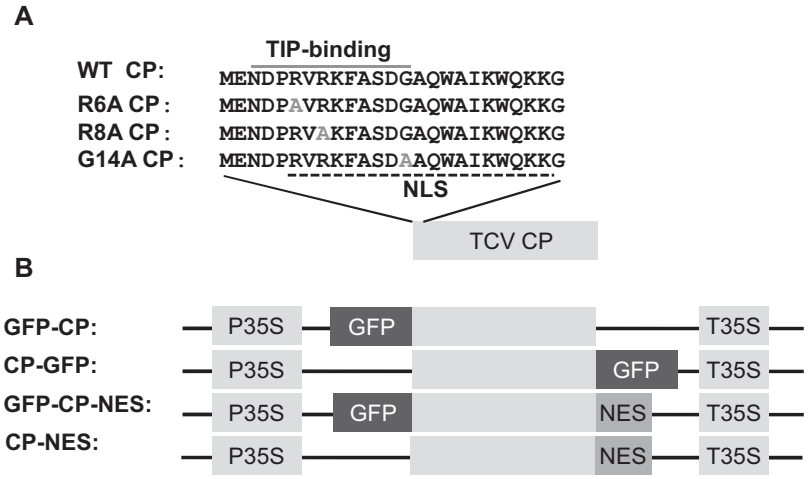

Fig. 1. Putative nuclear localization signal of TCV CP. A. The sequence of the amino-terminal 25 AA of the R domain of TCV CP and mutants used in this study. Residues in the predicted nuclear localization signal (NLS) are shown along with the TIP binding region. Cellular localization prediction by PSORT (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.

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 et al. (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

2.1.1. 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.

2.1.2. 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.

2.1.3. 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.

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