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A spectrum of HRT-dependent hypersensitive responses elicited by the 52 amino acid N-terminus of turnip crinkle virus capsid protein and its mutants

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Abstract

The capsid protein (CP) of turnip crinkle virus (TCV) is the elicitor of hypersensitive response (HR) and resistance mediated by the resistance protein HRT in the Di-17 ecotype of Arabidopsis. Here we identified the N-terminal 52-amino-acid R domain of TCV CP as the elicitor of HRT-dependent HR in *Nicotiana benthamiana*. Mutating this domain at position 6 (R6A), but not at positions 8 (R8A) or 14 (G14A), abolished HR in *N. benthamiana*. However, on Di-17 Arabidopsis leaves only R8A R domain elicited visible epidermal HR. When incorporated in infectious TCV RNAs, R8A and G14A mutations exerted dramatically different effects in Di-17 plants, as R8A caused systemic cell death whereas G14A led to complete restriction of the mutant

virus. This continual spectrum of HR and resistance responses elicited by various R domain mutants suggests that the CP–HRT interaction could be perturbed by conformational changes in the R domain of TCV CP.

The capsid protein (CP) of turnip crinkle virus (TCV) plays multiple roles in TCV–host plant interactions. In addition to its role in virus assembly, cell-to-cell and systemic movement, and suppression of RNA silencing (Hacker et al., 1992; Cohen et al., 2000; Li et al., 1998; Cao et al., 2010; Laakso and Heaton, 1993; Qu et al., 2003; Thomas et al., 2003), TCV CP is also responsible for eliciting TCV-targeting hypersensitive response (HR) and resistance in the Arabidopsis ecotype Di-17 that encodes HRT, a nucleotide-binding leucine-rich repeat (NB-LRR) resistance (R) protein (Dempsey et al., 1997; Cooley et al., 2000; Kachroo et al., 2000). Interestingly, this defense-triggering property of TCV CP appears to be dependent on the integrity of its N-terminus, as a number of single amino acid (AA) substitutions within this region were found to severely perturb the HRT-mediated HR and resistance (Ren et al., 2000; Zhao et al., 2000). An intimately related observation is that TCV CP physically interacts with TIP, an Arabidopsis NAC transcription factor (Ren et al., 2000). Intriguingly, single AA CP mutants that perturbed the induction of HRT-mediated defense also were compromised in their ability to interact with TIP (Ren et al., 2000). These earlier results led to the hypothesis that HRT serves as the guard for TIP by sensing the disruption of TIP structure/function by TCV CP and mounting a defense response to neutralize this disruption. Indeed, a follow-up study found that transiently expressed TIP normally localizes to cell nuclei in *Nicotiana benthamiana*, but this nuclear localization was disrupted by the co-expression of TCV CP (Ren et al., 2005). It should be noted that the role of TIP in HRT-mediated TCV resistance was discounted by a later study showing that the resistance response did not require the function of TIP (Jeong et al., 2008). Perhaps the most surprising finding of these earlier studies is that the CP–TIP interaction only required a very short N-terminal region of TCV CP (as short as 25 AAs of CP N-terminus), raising the interesting question of whether the same CP region might be sufficient for eliciting HR.

In the current study, we established that the N-terminally located, 52-AA long RNA-binding (R) domain of TCV CP induced robust, HRT-dependent HR indistinguishable from the full length CP in *N. benthamiana* leaves. Notably, two single AA mutants of CP, in which the arginine residue at position 8 and glycine residue at position 14 were changed to alanine (R8A and G14A,

respectively), also elicited strong HR in *N. benthamiana* leaves. Reexamination of TCV mutants containing the R8A and G14A mutations, referred to as TCV-R8A and TCV-G14A, in Di-17 plants revealed stark differences in their interplay with the HRT-mediated defense cascade. These results implies that the structural flexibility of the CP R domain plays a critical role in conditioning a continuum of resistance response, leading to dramatically different infection outcomes.

We first sought to test a number of CP fragments that correspond to well-defined structural domains of TCV CP for their ability in inducing HRT-dependent HR in *N. benthamiana* leaves. As shown in Fig. 1A, the 351 AA TCV CP can be divided into five structural domains: the 52 AA R domain, a 29 AA arm (A) linking R domain to the 162 AA surface (S) domain, a 5 AA hinge (H) permitting the outward bending of the 103 AA protruding (P) domain at the C-terminus. The N-terminal R domain was shown previously to interact with TIP *in vitro* (Ren et al., 2005). cDNAs of the R, RA, S, P and SHP domains of CP were cloned into the binary plasmid pPZP212 (Hajdukiewicz et al., 1994) to produce constructs ΔASHP, ΔSHP, ΔRAHP, ΔRASH, and ΔRA, respectively, all of which sandwiched by the 35S promoter and terminator (P35S and T35S) of cauliflower mosaic virus (Fig. 1A). Another construct designed to express the HRT protein tagged with a Myc epitope at its C-terminus (HRT-Myc) was also produced (Fig. 1B). These constructs were subsequently mobilized into *Agrobacterium tumefaciens* strain C58C1 for transient expression in *N. benthamiana via Agrobacterium* infiltration (agro-infiltration). *N. benthamiana* plants were reared in growth chambers set at 22 °C, 14 h light, and leaves of 4 week old plants were subjected to agro-infiltration with the concentration of *Agrobacterium* suspensions adjusted to OD600 = 1.0. In addition, an *Agrobacterium* strain harboring a construct designed to express the P19 silencing suppressor of tomato bushy stunt virus (TBSV) was included in most infiltrations to counteract RNA silencing (Figs. 1B and 2). As shown in Fig. 1B, co-expression of TCV CP and HRT-Myc elicited strong, rapid HR that caused the collapse of the infiltrated area by 3 days after agro-infiltration (3 DAI; top left panel). Similar HR was also observed when ΔASHP or ΔSHP was co-infiltrated with HRT, indicating that the R domain is the primary trigger of HRT-dependent HR in *N. benthamiana* leaves. By contrast, CP truncations lacking the R domain (ΔRAHP, ΔRASH and ΔRA) failed to elicit any visible HR even if the infiltrated plants were maintained for up to 7 days (Fig. 1B). Note that previous studies demonstrated that all these truncated forms of CP could accumulate to detectable levels in infiltrated *N. benthamiana* leaves in the presence of a silencing suppressor (Choi et al., 2004; Cao et al., 2010). Together these experiments demonstrate that in *N. benthamiana* cells, transiently expressed R domain of TCV CP readily elicit HRT-dependent HR.
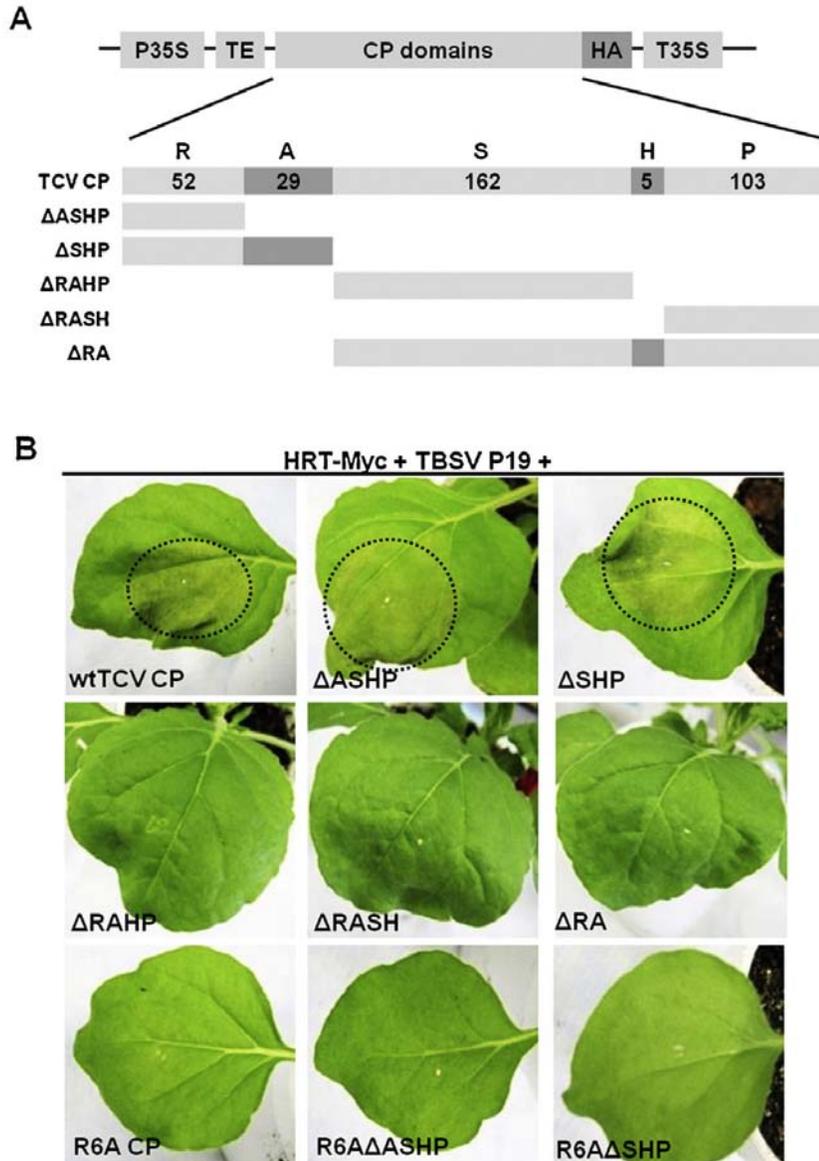
Fig. 1. HRT-dependent HR elicitation by TCV CP and its structural domains as assayed using transient expression in *N. benthamiana* leaves. (A) cDNAs of full length TCV CP as well as its various truncated forms were cloned into a binary vector (pPZP212), sandwiched by the 35S promoter (P35S) and terminator (T35S) of cauliflower mosaic virus. TE: translational enhancer derived from tobacco etch virus. HA: an HA epitope tag fused to the C-termini of all CP fragments. R, A, S, H, and P denote the RNA-binding, arm, surface, hinge, and protruding domains of TCV CP. Their corresponding sizes (in numbers of AAs) were shown in the corresponding boxes. (B) HR induction by TCV CP and its derivatives in *N. benthamiana* leaves. All constructs were delivered into *N. benthamiana* leaves with agro-infiltration. A construct that expresses the HRT R protein was co-delivered in all infiltrations (same for Fig. 2). Infiltrated leaves were photographed at 3 DAI.

We previously identified a number of single AA mutants within the TCV CP R domain that abolished both CP–TIP interaction and CP's ability to elicit HRT-mediated resistance in Di-17 plants (Ren et al., 2000, 2005). To test if such mutants also become incapable of eliciting HRT-dependent HR in *N. benthamiana* leaves, we first examined the R6A mutant of CP, in which the arginine residue at the position 6 of TCV CP was mutated to alanine, for its ability to induce HR in agro-infiltrated *N. benthamiana* leaves. As shown in Fig. 1B, bottom three panels, neither full length R6A CP, nor its R domain (R6AΔASHP and R6AΔSHP) elicited HR in *N. benthamiana* leaves. This result is consistent with our previous failure to observe resistance responses in Di-17 plants infected with the TCV-R6A mutant.

We next assessed the HR-eliciting potential of other R domain mutants containing single AA substitutions using the *N. benthamiana* transient expression system. This became necessary because a careful reevaluation of a number of previously reported TCV mutants with single AA mutations in the R domain of CP revealed that they caused very different symptoms in both inoculated and systemic leaves (IL and SL) of Di-17 plants (Donze, 2011). For instance, both TCV-R6A and TCV-R8A evaded the HRT-mediated resistance but caused distinct systemic symptoms. While TCV-R6A systemically invaded Di-17 without inducing systemic cell death, TCV-R8A induced systemic cell death and caused severe systemic leaf collapse that eventually led to death of the infected plants. In contrast, TCV-G14A elicited micro-HR on IL, but failed to cause systemic infections in any of the inoculated plants (Donze, 2011). Considering that wt TCV could overcome HRT-mediated resistance in 10–20% of Di-17 plants, the complete lack of systemic spread by TCV-G14A in Di-17 suggests that the G14A change enhanced the HR-eliciting potential of TCV CP (Dempsey et al., 1993).

To determine whether the contrasting disease symptoms caused by these mutants could be explained by the varying HR-eliciting potentials of the mutant CPs, we tested the transiently expressed CPs of R6A, R8A, and G14A side-by-side for their abilities to elicit HRT-dependent HR in *N. benthamiana* leaves. Visible HR developed as early as 2 DAI on the G14A CP-expressing leaves, followed shortly by the R8A CP-expressing leaves, and then by wt CP-expressing leaves at 3 DAI (Fig. 2A). R6A CP-expressing leaves were HR-free for the duration of the experiments (7 DAI). Notably, the G14A CP-elicited HR was not only fast developing, but also more extensive, eventually leading to a complete collapse of the infiltrated areas. These data appears to suggest a correlation between the speed of HR development and the effectiveness of systemic resistance. Similar to wt TCV CP, the HR-elicitation by R8A and G14A CPs was also mapped to their R domains in which the mutations are located. As shown in Fig. 2B, both R8A-ΔASHP and G14A-ΔASHP induced more pronounced HR than their
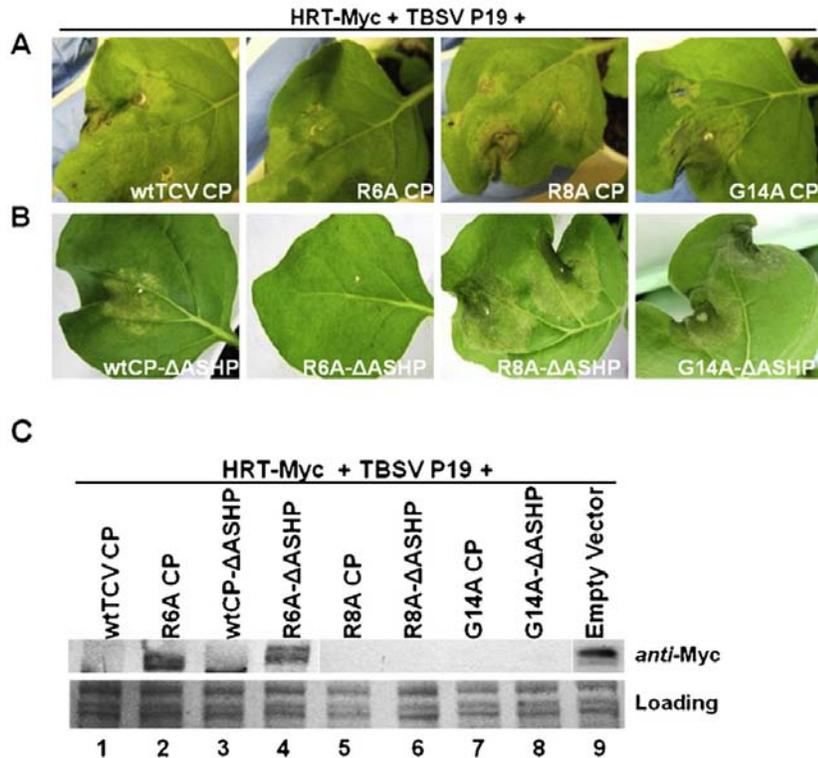
Fig. 2. HRT-dependent HR induction by three single AA mutants of CP, as well as their corresponding R domains, in *N. benthamiana* leaves. (A) HR induction by TCV CP and its R6A, R8A and G14A mutants. (B) HR induction by the R domains of TCV CP and its mutants. Infiltrated leaves were photographed at 3 DAI. (C) Western blot analysis of Myc-tagged HRT from protein extracts of the leaves shown in (A) and (B). The coomassie blue-stained gel was included at the bottom serving as the loading control. Infiltrated leaves were harvested at 2 DAI for Western blot analysis.

wildtype counterpart (wtCP-ΔASHP), thus clearly demonstrating that the R domain alone was responsible for triggering HRT-dependent HR, and the same likely holds true in TCV-resistant Di-17 plants.

It should be noted that potent induction of HR by the R8A CP and its R domain is somewhat inconsistent with our previous report that single AA mutations between residues 3–13 in the CP R domain compromised the ability of TCV CP to induce HR and resistance in Di-17 plants (Ren et al., 2000, 2005; also see later). To further corroborate these observations, the agro-infiltrated *N. benthamiana* leaf patches were harvested prior to HR development and subjected to Western blot analysis (ECL Plus, GE Healthcare Life Sciences, Piscataway, NJ) to evaluate the levels of HRT protein. This is accomplished using an anti-Myc antibody (9E10, Santa Cruz Biotechnology) as the

HRT-expressing construct contained a C-terminally fused c-Myc epitope tag. As shown in Fig. 2C, lane 9, HRT-Myc accumulated predominantly as a clearly detectable band of *ca.* 135 kDa. However, HRT was degraded to smaller sized smears upon co-expression with either wtTCV CP or wtCP-ΔASHP which is the R domain only (Fig. 2D, lanes 1 and 3). In contrast, HRT-Myc remained largely intact in the presence of R6A CP or R6A R domain (lanes 2 and 4). This correlation strongly suggests that HR is accompanied, or even preceded by HRT degradation, a phenomenon also observed with HR mediated by another NB-LRR protein (Boyes et al., 1998). Strikingly, co-expression of R8A and G14A CPs, as well as their R domains, led to a complete loss of HRT signal, suggesting that they induce even faster degradation of HRT than wt CP (lanes 5–8). These results are highly consistent with a definitive role of R8A CP, through its R domain, in HR induction.

To further determine whether R domain of CP is sufficient to induce HR in Di-17 Arabidopsis, we then created wtTCV, R6A, R8A, and G14A derivatives in which the CP was truncated to R domain only, designated wtTCV-ΔASHP, TCV-R6AΔASHP, TCVR8AΔASHP, and TCV-G14AΔASHP, respectively. Previous studies showed that TCV mutants lacking an intact CP could replicate in ILs of Arabidopsis plants but could not move systemically, primarily due to the disruption of the silencing suppressor function of CP (Cao et al., 2010). Consistent with these earlier findings, none of the R domain mutants caused visible systemic symptoms (data not shown). We first confirmed the viability of each of these mutants in the TCV-susceptible Col-0 ecotype of Arabidopsis by subjecting the ILs to Northern blot hybridizations with a TCV-specific probe. As shown in Fig. 3A, while the accumulation levels of all of ΔASHP mutants were drastically lower than their counterparts expressing intact CPs (compare lanes 1, 3, 5, 7 with 2, 4, 6, 8 respectively), it was possible to detect both genomic (g) as well as subgenomic (sg) RNAs of these mutants. These results indicated that all of these mutants are replication-competent in Col-0 Arabidopsis cells.

The accumulation levels of both full length mutants and their R domain-expressing derivatives (ΔASHP mutants) were notably different in ILs of Di-17 plants. As expected, both TCV-R6A and -R8A accumulated to similar levels in Col-0 and Di-17 ILs (compare lanes 3 and 5 in Fig. 3A and B), suggesting that these two mutants successfully subverted the HRT-mediated TCV resistance in ILs of Di-17. Additionally, the fact that wt TCV accumulated to much lower levels than TCV-R6A and -R8A in Di-17 ILs is consistent with its active containment by HRT-mediated resistance (Fig. 3B, lane 1). Strikingly, the replication of TCV-G14A was below the detection limit of our procedure (Fig. 3B, lane 7). When compared with wt TCV, it is obvious that the G14A change in CP R domain rendered this mutant hypersensitive to HRT resistance. In summary, the Northern blot results confirmed previous
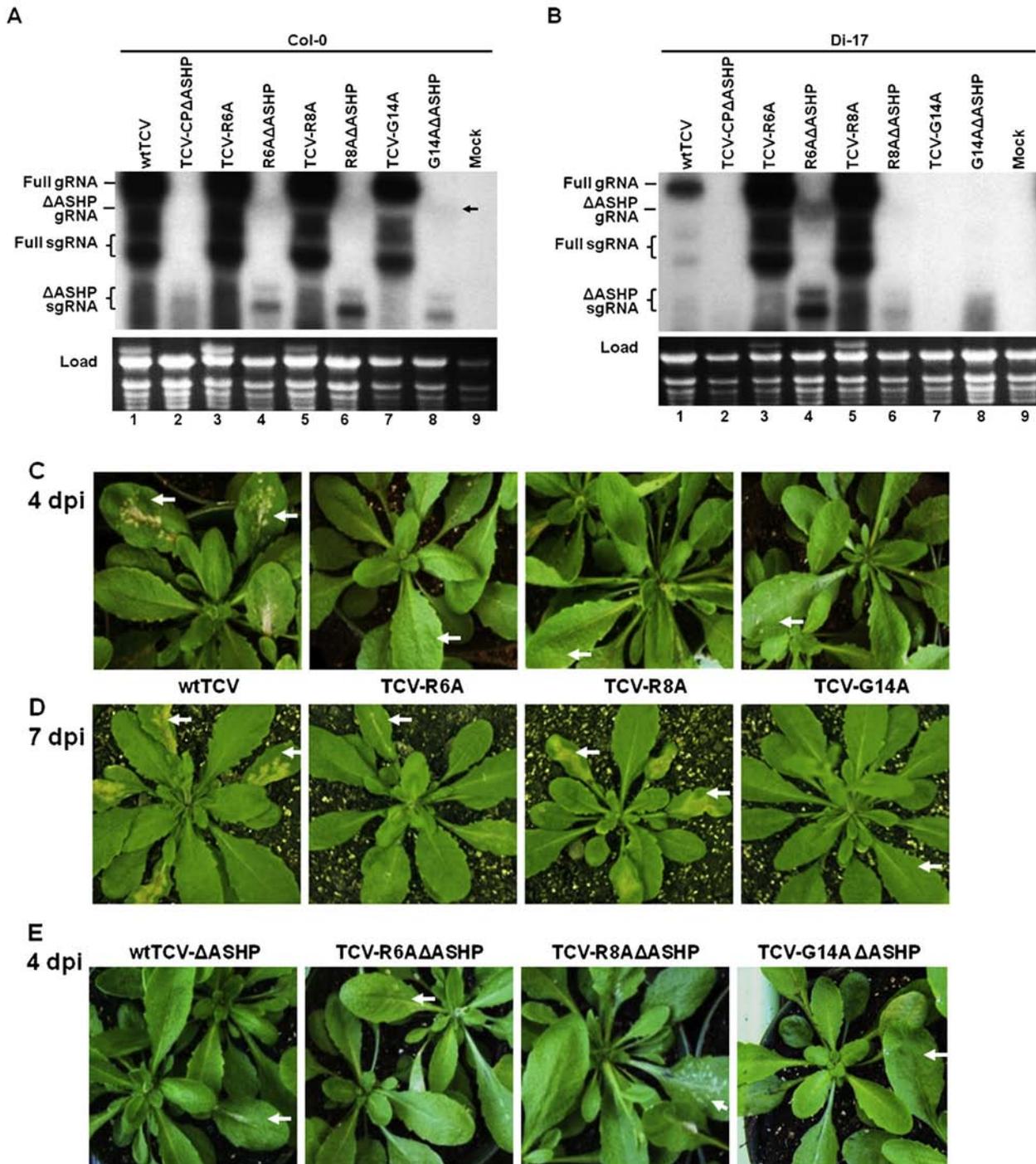
Fig. 3. Assessment of HR elicitation by TCV and its mutants in inoculated Arabidopsis leaves (ILs). All inoculations were initiated with *in vitro* transcripts of TCV and its mutants. (A) Replicability of the mutants in TCV-susceptible Col-0 ecotype of Arabidopsis as assessed with Northern blot hybridizations. Total RNA was extracted from ILs at 4 days post-inoculation (4 dpi). A ^{32}P-labeled DNA oligo complementary to the 3′ UTR of TCV was used as a probe. (B) Accumulation levels of (*continued*)

observations that identified TCV-R6A and -R8A as resistance-breaking mutants, and TCV-G14A as the one triggering extreme resistance (Ren et al., 2000, 2005; Donze, 2011).

We next examined the ILs of Di-17 plants for HR development. While well-developed HR was observed with wt TCV inoculations at 4 dpi (3C, left panel), it was absent on leaves inoculated with TCVR8A nor TCV-G14A (Fig. 3C, third and fourth panels). This result is in contrast with the rapid HR induced by R8A and G14A CPs in *N. benthamiana* leaves (Fig. 2A and B). Since the viral RNA of the TCV-G14A mutant was undetectable, we reason that the G14A CP could have elicited extreme resistance accompanied by microscopic HR invisible to naked eyes (Donze et al., 2014). Accordingly, transient overexpression of G14A CP and HRT in *N. benthamiana* leaves could have amplified the extreme HR manifested in single cells of virus-infected plants. In fact, similar situation has been documented for the Rx NB-LRR protein conferring resistance to Potato virus X (Bendahmane et al., 1999, 2002) and RPS2 NB-LRR protein conferring resistance to *Pseudomonas syringae* (Tao et al., 2000).

Although TCV-R8A inoculated leaves did not develop HR at 4 dpi, by 7 dpi these leaves developed foci of dead cells indicative of HR (Fig. 3D). However, when compared to HR foci induced by wt TCV, the TCV-R8A-induced, delayed HR appeared to be less penetrating but also less discrete. Indeed, it seemed that each IL contained one large focus of irregular shape and faint boundary, seemingly expanding toward the primary vascular bundle (Fig. 3D, third panel). These results suggest that the R8A-elicited HR in Di-17 leaves, rather than containing the virus, expanded along with the replicating virus. This led us to hypothesize that the R8A-associated HR was induced only after a substantial extent of TCV-R8A multiplication. Importantly, Di-17 infections with R domain only TCV mutants are consistent with this hypothesis. As shown in Fig. 3E, neither wtTCV-ΔASHP nor TCV-G14AΔASHP caused visible HR (first and fourth panels). This would be expected considering both of these mutants are much weaker than their full CP counterparts, hence would be restricted in microscopic foci by HRT-mediated resistance. Notably,

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various mutants in Di-17 plants as assessed by Northern blot hybridization. (C) Images of Di-17 plants inoculated with wt TCV, TCV-R6A, -R8A, and -G14A, taken at 4 dpi. White arrows denote ILs. HR was visible on ILs inoculated with wt TCV transcripts. (D) Images of Di-17 plants inoculated with wt TCV, TCV-R6A, -R8A, and -G14A, taken at 7 dpi. White arrows denote ILs. In addition to ILs containing wt TCV, R8A-inoculated leaves now showed vein-aligned, diffused foci of cell death. (E) Di-17 plants inoculated with *in vitro* transcripts of TCV constructs in which the CP of wt TCV, TCV-R6A, -R8A, and -G14A was truncated to R domain only (the ΔASHP mutants). Images were taken at 4 dpi. White arrows denote ILs.

TCV-R8AΔASHP did elicit visible, but less penetrating HR on Di-17 ILs. This would be expected if in these infections the TCV-R8AΔASHP mutant was able to spread to neighboring cells before the mutated R domain triggers HR. However, due to the truncation of the silencing suppressor, the mutant virus was weaker and hence was effectively contained by host defense in discrete foci.

How do we then reconcile these results with R8A (both full CP and R domain)-induced HR in *N. benthamiana* leaves? We speculate that HRT-dependent HR triggered by R8A CP behaved similarly in *N. benthamiana* and Di-17 Arabidopsis cells: in both cases probably only a small fraction of R8A CP was capable of HR induction. In Di- 17 cells, the HR-eliciting R8A fraction reached the threshold needed for HR induction only after abundant CP was synthesized by viral replication. On the other hand, in agro-infiltrated *N. benthamiana* cells such a threshold was reached sooner because of the greater abundance of CP mRNAs synthesized with the help of the powerful P35S promoter in the construct.

Based on the new insights revealed through the current study, we propose a new model to account for the varying degrees of HR and defense responses elicited by TCV and its various mutants. This model proposes that the R domain of TCV CP assumes at least two alternate conformations in infected cells, only one of which elicits effective HR. This is consistent with previous structural studies that found the R domain to be in a disordered state (Carrington et al., 1987). Conformational flexibility of the CP R domain could explain why wt TCV could escape the HRT-mediated resistance in 10–20% of Di-17 plants (*e.g.* Wobbe et al., 1998; Kachroo et al., 2000) – the alternate, non-HR-eliciting conformation could have dominated in those plants. Accordingly, various mutants could tilt the conformational balance, leading to the manifestation of a continuous spectrum of HR and defense responses. Specifically, R6A could have stabilized the non-HR-eliciting conformation, whereas G14A could have fixed the R domain to the HR-eliciting conformation. On the other hand, the R8A substitution could have diminished – but did not eliminate – the HR-eliciting conformation in the mixture. As a result, the HR-eliciting conformation was initially below the threshold needed for HR elicitation but eventually increased to this threshold as more CPs accumulated through viral multiplication.

This model not only explains the seemingly contradictory observations by us and others concerning TCV–Di-17 interactions, but also predicts a new layer of dynamics in other plant–pathogen interactions. Conformational flexibility of pathogen effector proteins affords pathogens with a selective advantage as it establishes a buffer zone from which resistance-escaping mutant could be quickly evolved. With the recognition of structural flexibility of pathogen effectors, it will be important for researchers to determine the

effector conformation most competent in inducing defense response. The mutagenesis studies conducted by our labs may offer some valuable lessons as such mutagenesis could lead to fixation of a certain conformation.

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