Plant Pathol. J. 19(1) : 19-23 (2003)

Symposium

The 5th Molecular Plant-Microbe Interactions

December 6, 2002, Daejeon, Korea

HRT-mediated *Turnip crinkle virus* Resistance in *Arabidopsis*

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(Received on November 20, 2002; Accepted on January 17, 2003)

Turnip crinkle virus **(TCV) inoculation onto resistant *Arabidopsis* ecotype Dijon (Di-17) leads to a hypersensitive response (HR) on the inoculated leaves. A dominant gene, *HRT*, which confers an HR to TCV, has been cloned from Di-17 plants by map-based cloning. *HRT* is a LZ-NBS-LRR class resistance gene and it belongs to a small gene family that includes *RPP8*, which confers resistance to *Peronospora parasitica* Emco5. Outside of the LRR region, HRT and RPP8 proteins share 98% amino acid identity while their LRR regions are less conserved (87% identity). *HRT*-transformed *Arabidopsis* plants developed an HR but generally remained susceptible to TCV due to a dominant *RRT* allele, which is not compatible with resistance. However, several transgenic plants that over-expressed *HRT* much higher than Di-17 showed micro-HR or no HR when inoculated with TCV and were resistant to infection. Both the HR and resistance are dependent on salicylic acid but independent of NPR1, ethylene, or jasmonic acid. *Arabidopsis* plants containing both TCV coat protein gene and HRT developed massive necrosis and death in seedlings, indicating that the TCV coat protein is an avirulence factor detected by the HRT.**

Keywords **:** HR, HRT, R gene, RPP8, SA, TCV.

Recognition of a diverse range of pathogens, followed by an appropriate defense response, is crucial for the survival of plants. The defense responses are often activated by a gene for gene interaction between a specific plant resistance (*R*) gene and a corresponding pathogen avirulence (*avr*) gene. Avr-R interactions lead to activation of various host defense responses, including a specialized type of programmed cell death known as a hypersensitive response (HR). Accumulating evidence indicates that *R* gene products function either directly as the receptor for the products of *avr* genes (Bent, 1996; Ellingboe, 1980; Yang et al., 1997) or recognizes the Avr factor indirectly through a co-receptor (Dixon et al., 1998). To date, direct interaction between an R protein and an Avr factor has been demonstrated only for the tomato Pto and the *Pseudomonas syringae* AvrPto proteins (Scofield et al., 1996; Tang et al., 1996), and between the rice Pi-ta and the *Magnaporthe grisea* Avr-Pita proteins (Jia et al., 1999).

The R proteins are either transmembrane or intracellular proteins that are presumed to initiate signal transduction cascades upon ligand binding (Fig. 1). Many *R* gene products share structural motifs, indicating that similar pathways might control resistance to diverse pathogens. To date, over 20 *R* genes have been identified and 5 classes are recognized: intracellular proteins with a nucleotide binding site (NBS), a leucine-zipper motif and a leucine-rich repeat (LRR) domain; intracellular NBS-LRR proteins with a

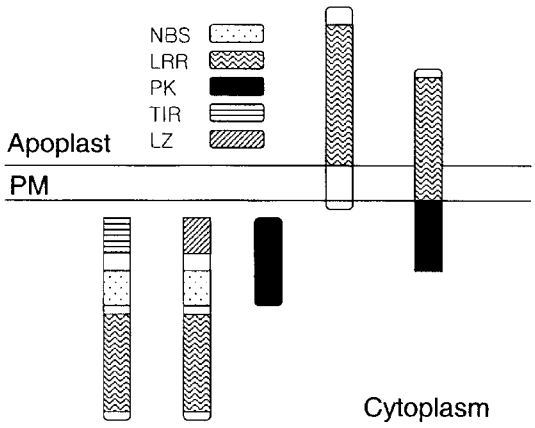

Fig. 1. Modular composition and predicted location of R protein classes. NBSLRR proteins are predicted to encode cytoplasmic receptor molecules. Abbreviations: LZ, leucine zipper; PK, protein kinase; LRR, leucine-rich repeat domain; NBS, nucleotide-binding site; PM, plasma membrane; TIR, Toll/interleukin-1-receptor.

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region of similarity to the cytoplasmic domain of mammalian IL-1 receptor (IL-1R) and the *Drosophila* Toll proteins (i.e. the TIR [Toll/IL-1R] domain); intracellular protein kinases (PKs); proteins with an LRR domain that encodes membrane-bound extracellular proteins; and receptor-like kinases (RLKs) with an extracellular LRR domain (Martin, 1999).

Many of the characterized *R* genes encode cytoplasmic proteins with NBS-LRR domains. The presence of an NBS region, also present in several ATP- and GTP-binding proteins, suggests that these proteins may play a role in the activation of a kinase or as a G protein (Martin, 1999). Most variation among R genes occurs within the LRR, particularly within the xxLxLxx, where L corresponds to conserved leucines (or other aliphatic amino acids) and x represents any amino acids, motif of the repeat units. This structure in the different repeats is thought to fit together to form a solvent-exposed parallel β sheet and is available for interaction with potential ligands (Kobe and Deisenhofer, 1995; Parniske et al., 1997). Indeed, in several plant-pathogen systems, sequence variation in the LRR has been shown to be responsible for different recognition or resistance specificities (Botella et al., 1998; Ellis et al., 1999; Parniske et al., 1997; Thomas et al., 1997; Wang et al., 1998;). However, other regions of the R protein, such as the TIR of the flax L protein (Ellis et al., 1999), may also contribute to recognition specificity.

As a consequence of an HR, a systemic signal is released from the point of infection that induces a secondary resistance response, known as systemic acquired resistance (SAR; Ryals et al., 1996). SAR is characterized by an increase in endogenous SA, transcriptional activation of the *PR* genes [*PR-1*, *BGL2* (*PR-2*) and *PR-5*], and enhanced resistance to a broad spectrum of virulent pathogens. SA is a necessary and sufficient signal for SAR because removing SA through the ectopic expression of salicylate hydroxylase (encoded by the bacterial *nahG* gene) blocks the onset of SAR (Gaffney et al., 1993). Jasmonic acid (JA) and ethylene (ET) have been shown to be involved in induced systemic resistance, which is activated by the nonpathogenic root-colonizing bacterium *Pseudomonas fluorescens* (Pieterse et al., 1996). Induced systemic resistance is independent of salicylic acid (SA), does not involve expression of *PR-1*, *PR-2*, or *PR-5*, and is blocked in *etr1*, *ein2*, *coi1*, and *jar1* mutants (Pieterse et al., 1996; Pieterse et al., 1998). Further evidence that JA plays an important role in plant defense was provided by the observation that methyl jasmonate induces resistance in *Arabidopsis* to *Alternaria brassicicola* and *Botrytis cinerea* and that this induced resistance is blocked in the *coi1* mutant (Thomma et al., 1998).

In *Arabidopsis*, resistance to most viral pathogens does not involve an HR (Lee et al., 1994; Callaway et al., 1996). However, inoculation of *Turnip crinkle virus* (TCV) (Morris and Carrington, 1988) on plants from the resistant ecotype Dijon (Di-0 or Di-17) results in both an HR and the induction of *PR* gene expression (Dempsey et al., 1993, 1997; Simon et al., 1992; Uknes et al., 1993). In contrast, TCV-susceptible ecotypes, including Columbia (Col-0), fail to mount an HR, exhibit delayed and weak *PR* gene expression, and develop systemic disease symptoms (Dempsey et al., 1993; Li and Simon, 1990). Genetic analyses revealed that HR development is conferred by a single dominant gene termed *HRT* (for HR to TCV) (Dempsey et al., 1997). *HRT* also appears to be required for resistance to TCV infection; all of the HR progeny from crosses between resistant and susceptible ecotypes developed systemic disease symptoms. However, *HRT* alone may not be sufficient for complete resistance because many of the HR progeny also succumbed to infection. Analysis of the *HRT* signaling pathway has revealed that the HR and resistance to TCV are dependent on SA but independent of *NPR1*-, ethylene-, and JA-mediated defense signaling.

Turnip crinkle virus

TCV, the most thoroughly studied member of the *Carmovirus* genus, is a 30-nm icosahedral plant virus consisting of a single-stranded, positive-sense RNA genome (4,053 nt) and 180 sub-units of a 38-kDa coat protein (CP) (Carrington et al., 1989). Sequence analysis of the TCV genome revealed the presence of five open reading frames (ORFs), of which the 5′ proximal ORF (p28) and its read-through product (p88) are both essential for genome replication (White et al., 1995). The 3′ proximal ORF encodes the CP (Carrington et al., 1987). The two overlapping internal ORFs (p8 and p9) were both shown to be involved in the cell-to-cell movement process (Hacker et al., 1992). While, most ecotypes of *Arabidopsis*, including Columbia (Col-0), are susceptible to TCV infection, the Dijon (Di-0) ecotype is partially resistant (Simon et al., 1992). From this Di-0 ecotype, both a TCV resistant (Di-17) and a susceptible (Di-3) line were isolated (Dampsey et al., 1993). After infection of resistant Di-17 plants with TCV, necrotic lesions developed on the inoculated leaves and the virus is restricted to these lesions in most plants. *PR* gene expression can also be detected in both inoculated and uninoculated leaves of Di-17. In contrast, plants from the Di-3 line fail to develop an HR after TCV inoculation. PR gene expression is delayed and weak in these plants compared with that observed in Di-17 plants. Within one week of inoculation, Di-3 plants develop systemic disease symptoms which increase in severity until plant death (Dampsey et al., 1993).

Characterization of *HRT* Gene

HRT was obtained from TCV-resistant Di-17 plants by map-based cloning and shown to confer an HR to TCV in transgenic Col-0 plants (Cooley et al., 2000). *HRT* shares extensive sequence similarity with members of the *RPP8* gene family, which includes *RPP8* and *RPH8A* of *Ler*-0 and *rpp8c* and *K15* of Col-0. *HRT* also shares moderate similarity with a fifth member of the family, located in BAC MOK9 from Col-0. Of the various *HRT* homologs, only *RPP8* is known to be a functional *R* gene (McDowell et al., 1998). Like *RPP*8, *HRT* is predicted to encode a protein of 105 kD that contains a leucine zipper, an NBS, and an LRR. Outside of the LRR region, these proteins share 96% amino acid similarity. However, despite this strong similarity, these proteins do not provide redundant functions. *RPP8* does not confer TCV resistance in *Ler*-0 plants, and *HRT* does not impart *P. parasitica* resistance in Di-17 plants or in transgenic Col-0 plants. Thus, different members of the *HRT/RPP8* gene family are responsible for activating resistance to either a viral or an oomycete pathogen.

Similarly, *Rx* and *Gpa2* members recognize radically different pathogens. *Gpa*2, which confers resistance against the potato cyst nematode *Globodera pallida*, was found to be tightly linked in the potato genome to *Rx*, which is responsible for resistance against *Potato virus X* (PVX; van der Voort et al., 1999). Their cloning and molecular characterization indicate that they are highly similar at the amino acid level and thus form a single *R* gene family (Bendahmane et al., 1999)

Interaction Between HRT and TCV CP

To confirm whether TCV CP gene is avirulence factor, 35S::CP transgenic Col-0 plants were constructed and crossed with Di-17 plants or HRT-transformed Col-0 plants (Cooley et al., 2000). All of the F1 progeny carrying the CP constructs developed systemic necrosis and died, demonstrating that CP is the TCV avr factor detected by HRT. However, whether the HRT product directly interacts with TCV CP has yet to be determined. Three naturally occurring strains of TCV that breaks resistance on Di-17 were isolated by repeated passage in this ecotype. These hypervirulent strains do not elicit HR on Di-17 but cause severe systemic disease just as the wild type of the TCV strain does on susceptible ecotypes like Col-0. The mutations responsible for bypassing the Di-17 surveillance system reside in the N-terminus of the CP (Zhao et al., 2000). Interestingly, Ren et al. (2000) found that this same region of the CP is responsible for interacting with TIP, which specifically interacts with CP in yeast. TIP belongs to the developmentally important NAC family of proteins and acts as a transcriptional activator in yeast cells. Single amino acid replacements within this TCV CP domain that resulted in loss of the specific protein-protein interaction also led to loss of resistance. Thus, this TCV CP-TIP interaction may be required for the *HRT*-elicited resistant response. Whether *HRT* interacts with the transcriptional activator or if the interaction occurs before or after *HRT* action is unknown.

HRT-mediated Downstream Signaling

The *HRT* signaling pathway was analyzed by crossing Di-17 plants with SA-deficient NahG plants, as well as the *npr1, ndr1, eds1, pad4, etr1* and *coi1* signaling mutants (Kachroo et al., 2000). The *HRT* pathway was found to require SA, while Di-17 NahG plants failed to develop an HR and were susceptible to TCV. However, this SA-dependent pathway is independent of *NPR1*. NPR1 plays an essential role in the SA-mediated signal transduction pathway that leads to the activation of PR genes (Cao et al., 1994; Delaney et al., 1995; Glazebrook et al., 1996). Di-17 plants carrying recessive *npr1* alleles developed lesions and were TCV resistant. *PR-1* expression was reduced in the *npr1* background, indicating that both NPR1-depedent and -independent pathways mediate expression of this gene after TCV infection. Analysis of progeny from crosses between Di-17 and the ethylene- or jasmonate-insensitive mutants, *etr1* and *coi1-1*, respectively, revealed that neither signal is required for HR development or TCV resistance. Di-17 plants were then crossed with *ndr1, eds1*, and *pad4* mutants to determine whether these genes are involved in HRT signaling. Neither HR formation nor TCV resistance were affected by the *ndr1* mutation. HR formation also was unaffected by the *eds1-1* and *pad4-1* mutations. In view of these features, the TCV resistance pathway differs from the SA-dependent, *NPR*1-dependent and the SA-independent, ethylene- or JA-dependent pathways involved in activating resistance to bacterial and fungal pathogens. Strikingly, however, the *HR*T-mediated resistance pathway shares many similarities with the SA-dependent, ethylene-independent *N* gene-regulated pathway for TMV resistance in tobacco (Knoester et al., 1998; Murphy et al., 1999). These discoveries provide further evidence that viral resistance in plants is activated via a mechanism distinct from those used for other microbial pathogens.

Conclusion

Currently, control of insect-transmissible viral pathogens relies primarily on pesticide application to reduce the populations of insect vectors; little can be done to salvage virus-infected crops. Thus, it is increasingly important to identify novel methods to control viral pathogens. A few

chemicals capable of inducing viral resistance have been identified; however, they must be applied prior to infection. By elucidating the mechanisms through which plants perceive and resist viral infection, successful strategies for engineering and/or manipulating disease resistance may be developed.

At this time, only four viral *R* genes have been cloned; the tobacco *N* gene (Witham et al., 1994), the potato *Rx1* and *Rx2* paralogs (Bendhammane et al., 1999), and the *Arabidopsis HRT* gene (Cooley et al., 2000). All encode NBS-LRR proteins; however, the N-termini of *HRT* and *Rx1/2* are CC domains, while that of *N* is a TIR domain. Since no single R protein class mediates viral resistance, the features required to recognize viral pathogens remain unknown. Knowledge of the downstream signaling pathways leading to viral resistance is similarly limited. In tobacco, *N* gene-conferred resistance to TMV is mediated by an SA-dependent pathway and appears to involve a SHAM-sensitive signaling component (Gaffney et al., 1993). Similarly, *HRT* signaling pathway in *Arabidopsis* is dependent on SA. Epistatic analyses show that TCV resistance is independent of NPR1, NDR1, ETR1, COI1 and perhaps of EDS1 and PAD4. Continued analysis of the HRT-mediated pathway will provide ground-breaking insights into how plant resists viral pathogens.

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