Members of the Arabidopsis HRT/RPP8 Family of Resistance Genes Confer Resistance to Both Viral and Oomycete Pathogens

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Turnip crinkle virus (TCV) inoculation onto TCV-resistant Arabidopsis leads to a hypersensitive response (HR) controlled by the dominant gene HRT. HRT is a member of the class of resistance (R) genes that contain a leucine zipper, a nucleotide binding site, and leucine-rich repeats. The chromosomal position of HRT and its homology to resistance gene RPP8 and two RPP8 homologs indicate that unequal crossing over and gene conversion may have contributed to HRT evolution. RPP8 confers resistance to an oomycete pathogen, Peronospora parasitica. Despite very strong similarities within the HRT/RPP8 family, HRT and RPP8 are specific for the respective pathogens they detect. Hence, the HRT/RPP8 family provides molecular evidence that sequence changes between closely related members of multigene families can generate novel specificities for radically different pathogens. Transgenic plants expressing HRT developed an HR but generally remained susceptible to TCV because of a second gene, RRT, that regulates resistance to TCV. However, several transgenic plants that overexpressed HRT produced micro-HRs or no HR when inoculated with TCV and were resistant to infection. Expression of the TCV coat protein gene in seedlings containing HRT resulted in massive necrosis and death, indicating that the avirulence factor detected by the HRT-encoded protein is the TCV coat protein.

INTRODUCTION

To survive, plants must defend themselves against numerous pathogens. Some defenses are constitutive, such as various preformed antimicrobial compounds (Osbourn, 1996), whereas others are activated by recognition of pathogens (Hammond-Kosack and Jones, 1996; Yang et al., 1997). The key players in this recognition process include the product of a dominant or semidominant resistance (R) gene present in the plant and the corresponding dominant avirulence (Avr) factor encoded by or derived from the pathogen. Recognition of the Avr factor by the host plant initiates one or more signal transduction pathways that activate various plant defenses and thus compromise the ability of the pathogen to colonize the plant. In this gene-for-gene interaction, the prevailing thought is that the R protein either acts directly as the receptor of the Avr factor (Ellingboe, 1980; Bent, 1996; Yang et al., 1997) or recognizes the Avr factor indirectly through a coreceptor (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 (Scocfield et al., 1996; Tang et al., 1996) and between the rice Pi-ta and the Magnaporthe grisea AvrPita proteins (Jia et al., 1999).

An array of R genes that provide protection against viruses, bacteria, fungi, and oomycetes has been cloned from both monocots and dicots during the past 6 years (Staskawicz et al., 1995; Bent, 1996; Baker et al., 1997; DeWit, 1997). Many contain a nucleotide binding site (NBS). Often located closer to the N terminus of the R protein is either a leucine zipper or a TIR domain, which is similar to the intracellular C-terminal signaling domain of the integral membrane Drosophila Toll protein and the mammalian interleukin-1 receptor. Both the Toll protein and the interleukin-1 receptor are involved in signaling pathways that lead to activation of the defense responses to pathogens in Drosophila and mammals, respectively. Two R proteins have also been shown to contain a serine/threonine kinase domain.

In addition to these motifs, all but two R proteins involved in gene-for-gene interactions have a leucine-rich repeat (LRR) region. This domain consists of imperfect repeats of nine to >40 units, each of which is ~25 amino acids long. In the central region of each repeat is a β strand/β turn structure,
which is hypervariable and has the consensus sequence XX(L/X)LXXX, where L corresponds to conserved leucines (or other aliphatic amino acids) and X denotes the flanking hypervariable amino acids (Parniske et al., 1997; McDowell et al., 1998). This structure in the different repeats is thought to fit together to form a solvent-exposed parallel β sheet (Kobe and Deisenhofer, 1995). Such a solvent-exposed, hypervariable surface could facilitate interaction of the R protein with its cognate Avr factor (ligand) and could provide different recognition specificities for altered Avr factors (Parniske et al., 1997). Indeed, in several plant-pathogen systems, sequence variation in the LRR, particularly in the β strand/β turn motif, has been shown to be responsible for different recognition or resistance specificities (Hulbert, 1997; Parniske et al., 1997). 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.

Many R genes belong to tightly linked multigene families. For example, the tomato Cf-4/9 (Parniske et al., 1997; Thomas et al., 1997) and Cf-2/5 loci (Dixon et al., 1998), the Arabidopsis RPP1/10/14 locus (Botella et al., 1998), the flax M locus (Anderson et al., 1997), the lettuce Dm3 locus (Anderson et al., 1996; Meyers et al., 1998), and the maize Rp1 locus (Sudupak et al., 1993; Collins et al., 1999) all consist of genetically linked R genes that specify resistance to different pathovars or biotypes of the same pathogen. Recent studies on the maize Rp1 and tomato Cf-4/9 loci have suggested that some of the different R specificities to common rust fungus (Puccinia sorghi) or Cladosporium fulvum, respectively, were generated by recombination between different family members (Hulbert, 1997; Parniske et al., 1997; Collins et al., 1999). Frequently, different R genes and their associated family members are located in genetically linked clusters with unrelated R genes (Michelmore and Meyers, 1998). Clusters of R genes (or R gene families) may span as much as 20 centimorgans (cM) and specify resistance to divergent pathogens. Although sequence comparison of orthologs and paralogs within these families that make up an R gene cluster suggests that interallelic recombination and gene conversion serve as the predominant source of new specificities (Michelmore and Meyers, 1998), no evidence indicates that recombination between R gene families generates novel recognition specificities.

After an R gene–mediated recognition of pathogen attack, often various defense responses are activated, such as the accumulation of salicylic acid (SA), the expression of pathogenesis-related (PR) genes, and the development of a hypersensitive response (HR) on the inoculated leaves (Yang et al., 1997). The HR is defined by necrotic lesion formation at the site of infection; generally, it is thought to help prevent pathogen multiplication and movement by confining the pathogen to dead cells, where nutrients or critical host components for replication are limiting or absent. However, the HR is not always required for resistance. Arabidopsis carrying the dnd1 mutation fail to develop an HR but are still capable of resisting infection by P. syringae in a gene-for-gene manner (Yu et al., 1998). Subsequent to HR development, increases in SA content and PR gene expression are often detected in the upper, uninoculated leaves, coincident with the development of systemic acquired resistance to a secondary challenge by a broad spectrum of pathogens (Ryals et al., 1996; Dempsey et al., 1999).

In Arabidopsis, turnip crinkle virus (TCV) produces an HR 2 to 3 days postinoculation (DPI) in ecotype Dijon (Di-0 and Di-17) (Simon et al., 1992; Dempsey et al., 1993; Uknes et al., 1993). To date, all other ecotypes of Arabidopsis tested do not give an HR but allow systemic spread of the virus (Li and Simon, 1990). A dominant gene, HRT, which confers an HR to TCV, has been identified and mapped in the Di-17 line of Dijon to a region 2 cM telomeric of the DFR marker on chromosome 5 (Dempsey et al., 1997). Here, we report the cloning of HRT and show that this gene probably arose, at least in part, by unequal crossing over between progenitor genes related to RPP8 and RPH8A of the Landsberg erecta (Ler-0) ecotype. Given that RPP8 is responsible for resistance to the oomycete pathogen Peronospora parasitica, the RPP8/HRT gene family provides a rigorous demonstration that different members of the same family can mediate recognition of different pathogens.

**RESULTS**

**HRT Mapping and Cloning**

The region containing HRT was determined by screening an F2 mapping population of 1223 individuals obtained from a cross between plants from the resistant Di-17 line of the Dijon ecotype and the susceptible Columbia (Col-0) ecotype. Thirty-six recombinants were found, initially placing HRT in a region between two markers, DFR and mi83, on chromosome 5 (Figure 1A), a region in which several markers have been identified. Further analysis identified two recombinants that restricted HRT to a 220-kb region between the internal markers mi194 and 4RL. Using fragments of these markers as probes, we screened a bacterial artificial chromosome (BAC) library made from Di-17 plants and constructed a contig spanning mi194 and 4RL (Figure 1A). The four BACs that make up the contig between these markers were individually subcloned into the binary vector BIBAC2 (Hamilton et al., 1996) as 30- to 40-kb BamHI partial fragments. These subclones were then transformed into Col-0 plants to test for complementation, that is, whether they would produce an HR when inoculated with TCV. The entire region represented by the four BACs was thus tested, and two subclones (E2 and E9) from BAC 11E3 were observed to produce transgenic plants that developed an HR after TCV infection (Table 1). Additional subclones of the E2 fragment were made in the binary vector pBIN19, and after complementation tests as above, HRT was localized to a 5.2-kb
EcoRI fragment (E2-1; Figure 1A). This fragment contains a single open reading frame (ORF) interrupted by introns. A cDNA library made from TCV-inoculated Di-17 plants was initially screened for cDNAs that hybridized to E2 and subsequently to subclones of E2. One full-length cDNA was identified and designated H116. Sequence analysis revealed that H116 corresponded to the E2-1 fragment. This cDNA was cloned behind the cauliflower mosaic virus (CaMV) 35S promoter and transformed into Col-0 plants. These transgenic plants developed an HR when inoculated with TCV (Figure 2 and Table 1), confirming that this ORF is HRT.

Based on the sequence of H116, HRT was shown to encode a putative protein of 105 kD that contains an N-terminal leucine zipper, an NBS, and a C-terminal LRR region consisting of 14 imperfect repeats (Figure 3). Comparison of the HRT sequence with those in the GenBank database indicated that it shares extensive sequence similarities (91.5 to 92.2% identity) in amino acid level with two genes in the Ler-0 ecotype and two genes in the Col-0 ecotype (Figure 3). The two genes in Ler-0 are RPP8 and an adjacent homolog RPH8A; like HRT, both are located just telomeric of the agp6 marker (McDowell et al., 1998). RPP8 confers resistance to the oomycete pathogen P. parasitica; whereas the function of RPH8A is unknown. In Col-0, HRT shares extensive similarity with rpp8c (rpp8-Col), which does not confer resistance to P. parasitica. This gene may have arisen by unequal crossing over between progenitor genes related to RPP8 and RPH8A (McDowell et al., 1998). HRT also is highly similar in sequence to an ORF, termed K15, found in the Col-0 BAC K15N18. K15 is located 2.5 Mb telomeric of rpp8c; whether it serves as a functional R gene is not known.

Interestingly, the strong similarity between HRT and the RPP8 family of R genes is not restricted to their coding regions but also includes portions of their 5' and 3' flanking sequences and their introns (Figure 1B). Sequence comparison...
between HRT and the Ler-0 genes RPP8 and RPH8A revealed that the 2459-bp portion of the HRT gene 5’ of the LRR (starting at the beginning of the transcript and continuing into the ORF) shows the greatest similarity (96%) with the corresponding 5’ region of RPP8. Additionally, even more 5’ to this region, sequence identity with RPH8A is negligible beyond 271 bp but continues with RPP8 (93%). In contrast, the 3’ portion of the HRT gene exhibits the greatest similarity (95% identity) to the corresponding 3’ region of RPH8A (1601 bp from the beginning of the first LRR to the end of the transcript), and substantial sequence identity between HRT and RPP8 is lost at 439 bp from the 3’ end of the transcript. Strikingly, the chimeric structure of HRT and its flanking sequences are nearly identical to those of rpp8c from Col-0 (McDowell et al., 1998). The only major difference between these genes occurs in the 3’ untranslated region, where rpp8c contains two unique insertions and one deletion (data not shown). Largely because of the chimeric structure of rpp8c, McDowell et al. (1998) proposed that this gene arose from a recombination event between progenitor genes related to RPP8 and RPH8A. Analysis of seven insertion/deletion segments (indels) in the coding regions of RPP8, RPH8A, and rpp8c further suggested that the recombination breakpoint within rpp8c occurred in a 75-amino acid region within or just 5’ to the first LRR. Because HRT contains the same indels as rpp8c, and because the flanking sequences of both genes are almost identical, it seems likely that these genes arose from the same or a similar unequal crossing-over event between progenitor genes related to RPP8 and RPH8A.

High-stringency DNA gel blot analysis using the HRT-containing H116 cDNA clone as a probe indicated that at least one homolog of HRT exists in Di-17 plants (Figure 4). In addition to the 5.2-kb EcoRI fragment that contains HRT, a 12-kb EcoRI fragment was detected. The latter fragment also is present in TCV-resistant Di-0 plants and in TCV-susceptible Ler-0 and Di-3 plants (Di-3 was derived from the original Di-0 ecotype; Dempsey et al., 1993). Because DNA gel blot analysis of the BAC contig detected only a single band corresponding to the expected size of HRT (data not shown), this homolog must not be in the 220-kb region around HRT. Moreover, the 5.2-kb band containing HRT was not detected in either Ler-0 or Di-3 plants; instead, a slightly larger band (~5.5 kb) was observed. The hybridization pattern observed with EcoRI-digested Col-0 DNA was completely unrelated to those seen with Dijon or Ler-0 (Figure 4). Sequence data available from the Arabidopsis sequencing project indicate that the largest fragment (7.7 kb) contains rpp8c. The 6.5-kb fragment corresponds to K15, and the smallest fragment contains an ORF found in BAC MO9, which is located 3.5 Mb closer to the centromere of rpp8c. Comparison of HRT with the predicted protein sequence from an ORF in MOK9 showed 84% identity at the amino acid level, considerably less than in the other comparisons. The 12-kb fragment detectable in Di-17, Di-0, Di-3, and Ler-0 could be an allele of either K15 or the ORF in MO9.

**Table 1. Responses of Different Ecotypes and Transgenic Plants to Infection by TCV or P. parasitica Emco5**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>TCV&lt;sup&gt;a&lt;/sup&gt;</th>
<th>P. parasitica Emco5&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ler-0 wt&lt;sup&gt;c&lt;/sup&gt;</td>
<td>S</td>
<td>R</td>
</tr>
<tr>
<td>Col-0 wt</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td>Di-0 wt</td>
<td>HR</td>
<td>R</td>
</tr>
<tr>
<td>Di-17 wt</td>
<td>HR</td>
<td>R</td>
</tr>
<tr>
<td>Di-3 wt</td>
<td>S</td>
<td>R</td>
</tr>
<tr>
<td>Col-0:E2</td>
<td>HR</td>
<td>S</td>
</tr>
<tr>
<td>Col-0:E9</td>
<td>HR</td>
<td>S</td>
</tr>
<tr>
<td>Col-0:E2-1</td>
<td>HR</td>
<td>S</td>
</tr>
<tr>
<td>Col-0::35S:HRT</td>
<td>HR</td>
<td>S</td>
</tr>
<tr>
<td>Ler-0::E2-1</td>
<td>HR</td>
<td>ND&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup>HR, hypersensitive response; S, susceptible, no HR but disease symptoms, including crinkling of the leaves and dropping of the bolts.

<sup>b</sup>R, resistant, no sporangiophores; S, susceptible, >10 sporangiophores per cotyledon. The number of sporangiophores was determined 5 DPI.

<sup>c</sup>wt, wild type.

<sup>d</sup>ND, not determined.

**Resistance to P. parasitica**

Given the nearly identical amino acid sequences of HRT and RPP8, we tested whether HRT, like RPP8, confers resistance to the Emco5 isolate of P. parasitica. Di-0, Di-3, and Di-17 plants were all resistant to Emco5 (Table 1). However, analysis of 20 recombinant F3 families from the original Di-17 × Col-0 mapping population indicated that resistance to P. parasitica Emco5 is not linked to HRT in Di-17 plants (data not shown). More probably, therefore, Di-17 plants (and also Di-0 and Di-3 plants) contain a yet uncharacterized gene related to HRT.

![Figure 2](image-url)
R gene that recognizes Emco5. Consistent with these results, transgenic Col-0 plants expressing HRT were as susceptible as the parental wild-type Col-0 plants to Emco5 (Table 1). None of the Col-0 plants transformed with genomic fragments E2, E9, or E2-1 or with the CaMV 35S promoter:HRT cDNA construct gained resistance to this pathogen. Conversely, the expression of HRT in transgenic Col-0 or Ler-0 plants conferred the ability to develop an HR after TCV infection (Table 1). Thus, HRT was necessary to confer recognition of TCV in Ler-0 and Col-0 plants but did not confer recognition of Emco5 in Col-0 plants. Therefore, despite being so similar, HRT and RPP8 do not share the same recognition function.

Expression of HRT

To determine whether HRT expression is altered by TCV infection, we performed quantitative reverse transcription-polymerase chain reaction (RT-PCR), using HRT-specific primers designed not to cross-hybridize with any potential transcripts from the various genes similar to HRT. Quantification was achieved by coamplification of a fragment containing part of HRT and a 150-bp insertion as an internal standard (see Methods). Total RNA was extracted from the leaves of mock- and TCV-inoculated Di-17 and Col-0 plants at 2 DPI. RT-PCR amplification revealed no substantial difference in HRT expression between mock- and TCV-infected Di-17 leaves (data not shown). The amounts of the RT-PCR products were 10- to 20-fold lower with RNA from Col-0 than with RNA from Di-17, arguing that the primers were indeed quite specific.

Analysis of Resistance in HRT-Expressing Transgenic Plants

Genetic analyses of the F1 and F2 progeny of crosses between Di-17 plants and TCV-susceptible ecotypes, including Col-0 and Nössen, indicate that HRT is necessary for both the HR and TCV resistance (Kachroo et al., 2000). In addition, a second locus, termed RRT, also regulates resistance. The rrt allele present in Di-17 is recessive to those found in susceptible ecotypes. Thus, it was not surprising that ~90% of the HRT transgenic Col-0 plants, although HR+, were susceptible to TCV. However, several lines of TCV-resistant transgenic plants were also detected, despite the presence of the dominant RRT allele from Col-0, which is not compatible with resistance. One possible explanation for this result is that the resistant lines might express HRT to very high amounts, which might overcome the negative influence of the RRT locus or substitute for the positive effect of the rrt allele on resistance. We therefore quantified HRT expression by RT-PCR and determined the resistance status by checking for disease symptoms and the presence of viral transcripts in the systemic tissues of TCV-infected transgenic plants. In addition, the development of an HR was monitored with trypan blue, which selectively stains dead or dying cells and thus facilitates the detection of very small lesions (micro-HR).

Because TCV inoculation involves abrasive wounding of the leaf, some staining (along the veins and at the base of trichomes) was always evident, even when an HR did not occur, such as in Col-0 plants (Figure 5) or in the mock-inoculated controls (data not shown). In contrast, the HR was visible as an expanded region of darkly stained cells surrounding the wound site (Figure 5, Di-17). By combining trypan blue analysis with HRT quantification and resistance assessments, we detected three categories of Col-0 transgenic plants. Approximately 90% of transgenic plants produced an HR, expressed PR-1 in high amounts, and allowed systemic spread and replication of the virus (Figure 5, HR). HRT expression in these plants was comparable with that seen in Di-17 plants. The second category consisted of two lines from among the 44 HRT-expressing transgenic lines tested. These lines were highly resistant to TCV, although they failed to produce a readily detectable HR, and no trypan blue staining occurred beyond the typical wounding response seen in Col-0 plants (Figure 5, no HR). Neither PR-1 expression nor TCV RNA could be detected in the uninoculated tissues of these plants, and HRT expression was much higher (three- to fivefold) than in Di-17 plants. This highly resistant phenotype was stable, being seen also in the T3 and T4 generations.

The third class of plants exhibited intermediate resistance. Like the highly resistant transgenics, they failed to develop an easily detectable HR; however, a low percentage of their progeny was susceptible to TCV. The different transformed lines produced progeny exhibiting different percentages of resistance, which was heritable and generally >95%. Trypan blue staining revealed a few darkly stained cells surrounding the initial infection sites, suggesting the formation of micro-HRs (Figure 5, micro-HR). In contrast to the darkly stained cells detected in mock- or TCV-infected Col-0 leaves, the dying cells associated with these micro-HRs were not associated with trichomes (Figure 5, Col-0 and no HR). The formation of micro-HRs suggests that the virus was able to replicate and spread to a very few cells in these plants. Moreover, the virus occasionally was able to spread beyond the micro-HR and was detected in the uninoculated leaves. In contrast, plants exhibiting the highly resistant phenotype appeared to localize the virus more efficiently. The uninoculated leaves of micro-HR–forming plants exhibited small amounts of PR-1 transcripts after infection (Figure 5, micro-HR), and HRT expression was intermediate, generally greater than the low levels detected in Di-17 plants but less than that observed in the highly resistant transgenics. Thus, although HRT expressed to the same extent as in Di-17 did not lead to resistance in transgenic Col-0 plants, high HRT expression generally correlated with resistance to viral spread and a reduction in lesion size or elimination of the HR entirely.
**Figure 3.** Amino Acid Alignment between HRT and Homologs.

The leucine zipper is indicated by asterisks, the NBS is highlighted by pound signs, and the LRRs are underlined and numbered. Note that HRT contains a second potential leucine zipper overlapping the NBS. The β strand/β turn structure within each LRR is indicated in boldface and un-
Interaction between TCV Coat Protein and HRT

Previous studies have suggested that the TCV coat protein (CP) is the Avr factor recognized in Di-0 and Di-17 plants (see Discussion). To test this possibility rigorously, we constructed transgenic Col-0 plants that expressed the CP under the control of the CaMV 35S promoter. Expression of the CP gene was verified by immunoblot analysis (data not shown). A CP-expressing Col-0 plant was then crossed with a Di-17 plant, with the expectation that if the CP is the Avr factor, its interaction with HRT should cause cell death and necrosis. Because a T₁ generation of a CP transgenic plant was used in this cross, all of the F₁ progeny should contain one copy of HRT and 50% should also contain the CP transgene. F₁ seeds were plated on kanamycin-containing medium to select for those carrying the CP transgene. Kanamycin-resistant and -sensitive seedlings were detected at a ratio of ~1:1. The kanamycin-sensitive seedlings failed to develop roots or initiate their first leaves; eventually, their cotyledons became chlorotic. In contrast, the kanamycin-resistant seedlings developed normally until ~2 days after initiation of the first leaf, but then suddenly they developed massive necrosis and died (Figure 6B). No such developmental abnormalities or necrosis was detected in seedlings from the CP-expressing Col-0 line used in the cross (Figure 6A). Thus, we conclude that the TCV CP is the Avr factor recognized by HRT in Di-17 plants. The F₁ progeny of a cross between CP-expressing and HRT-expressing Col-0 plants also exhibited massive necrosis and death, confirming the identity of the R protein and the Avr factor (Figure 6C).

DISCUSSION

HRT is a Member of the RPP8 Gene Family

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 (Figure 2 and Table 1). 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. Like RPP8, HRT is predicted to encode a protein of 105 kD that contains a leucine zipper, an NBS, and an LRR (Figure 3). 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 (Table 1). Thus, different members of the HRT/RPP8 gene family are responsible for activating resistance to either a viral or an oomycete pathogen.

The different resistance specificities of HRT and RPP8 probably result from variations between their LRRs. Both HRT and RPP8 contain LRRs consisting of 14 imperfect repeats with the consensus β strand/β turn motif XX(L)X(L)XXXX (Figure 3). A pairwise comparison of the five homologs indicated that they are almost equally divergent in this region; the rate of substitution in the seven hypervariable residues averaged 26.9%, nearly five times more than the rest of the protein combined. The lowest rate of substitution at these sites was between RPH8A and rpp8c (24.5%), and the greatest was between RPP8 and K15
Comparison of HRT and RPP8 showed an intermediate value of 27.5%. Presumably amino acid substitutions at these solvent-exposed sites would alter the specificity of the R protein–Avr protein interaction. Similar amino acid substitution rates were also seen with HRT adjacent to the $\beta$ strand/$\beta$ turn motif within several LRRs (Figure 3, LRRs 1, 4, 5, 8, and 11) and in two regions on the N-terminal side of the first LRR (amino acids 427 to 447 and 481 to 492), as was previously observed between the different RPP8 homologs (McDowell et al., 1998).

The ability of different members of the HRT/RPP8 gene family to confer resistance to unrelated pathogens bears some similarity to the tomato Mi gene, which confers resistance against two distinct pathogens, a nematode and an aphid (Milligan et al., 1998; Rossi et al., 1998). In addition, Rpm1 from Arabidopsis recognizes two very different Avr factors, AvrB4 and AvrRpm1 from P. syringae (Grant et al., 1995). However, these two cases differ from that of the HRT/RPP8 family in that both Mi and Rpm1 presumably have dual specificity for unrelated Avr factors. The HRT/RPP8 family is novel because it provides the first molecular evidence that different members of an R gene family confer recognition of very different pathogens. Rx and Gpa2 appear to be a second example of a family whose members recognize radically different pathogens. Gpa2, 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; J. Bakker, personal communication).

HRT appears to have been formed in part by an unequal crossing over between progenitor genes related to RPP8.

### Figure 5. Comparison of Lesion Size, TCV Spread, and PR-1 Expression with HRT Expression.

Inoculated leaves were stained with trypan blue to show the relative size of necrotic tissue at 3 DPI. RNA isolated from uninoculated (U) leaves at 6 DPI was analyzed for PR-1 induction, systemic spread of TCV, and HRT expression. PR-1 expression and TCV systemic spread were determined by RNA gel blot analysis probed with PR-1 or pTTTCV66, respectively. The amount of HRT expression (given in picograms at bottom) was determined by RT-PCR.

<table>
<thead>
<tr>
<th>Trypan blue stained</th>
<th>Col-0</th>
<th>HR</th>
<th>micro-HR</th>
<th>no HR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Di-17</td>
<td>0.5-0.9</td>
<td>3-10</td>
<td>8-15</td>
</tr>
</tbody>
</table>

### Figure 6. Massive Necrosis and Death of Seedlings Due to Expression of the TCV CP and HRT.

(A) Col-0::35S:CP transgenic seedlings.
(B) F1 plants of a cross between Col-0::35S:CP transgenic plants and Di-17.
(C) F1 plants of a cross between Col-0::35S:CP transgenic plants and Col-0 homozygous for the HRT transgene (Col-0::35S:HRT). Seeds were plated on media containing kanamycin. The smaller, chlorotic (light yellow) seedlings were kanamycin sensitive, whereas kanamycin-resistant seedlings showed extensive root and leaf growth. All necrotic seedlings showed both root and leaf growth before death. Arrows denote several of the necrotic, dying, kanamycin-resistant seedlings.
and RPH8A (Figure 1B; McDowell et al., 1998). Recombination also is thought to play a role in generating different recognition specificities to the common rust fungus within the Rp1 complex of maize (Collins et al., 1999). Apparently, recombination within R gene families may contribute novel specificities for detection both of variants of the same pathogen and of radically different pathogens.

**HRT and Resistance to TCV**

In this article and that of Kachroo et al. (2000), we establish that HRT is necessary but generally insufficient for resistance. Here, we show that ~90% of the HRT-transformed Col-0 plants developed an HR and expressed large amounts of PR-1 after TCV infection yet were still susceptible (Table 1 and Figure 5). In the accompanying article, we demonstrate the existence of a second gene, RRT, that regulates resistance to TCV (Kachroo et al., 2000). Whether this regulation is positive or negative is currently unclear; however, the allele present in TCV-susceptible Col-0 plants is dominant to the Di-17 allele. Thus, it was surprising that ~10% of the HRT-transformed Col-0 plants were TCV resistant. Interestingly, the transgenic lines that were resistant did not develop a normal HR. Trypan blue staining indicated that some of these lines developed micro-HRs, in which the number of dead cells were much fewer than those in a normal HR (Figure 5). In addition, two of the TCV-resistant transgenic lines exhibited neither a visible HR nor a micro-HR after TCV infection. This HR− resistance is reminiscent of the extreme resistance exhibited by potatoes carrying Rx. In Rx-containing potato plants, the rapid arrest of PVX replication in the initially infected cells appears to prevent development of an HR (Kohm et al., 1993; Bendahmane et al., 1999).

Analysis of HRT mRNA levels in the transformed lines revealed that the extent of HRT expression directly correlated with the extent of TCV resistance and was inversely proportional to lesion size. Plants of the HR− but extreme resistant lines expressed three- to fivefold more HRT than did Di-17 plants (Figure 5), whereas lines exhibiting a micro-HR and partial resistance had intermediate HRT expression. These results are consistent with data in the accompanying article (Kachroo et al., 2000), which suggest that resistance is influenced by the homozygous or heterozygous state of HRT. A greater than expected number of F2 progeny from a Di-17 × Col-0 cross were both heterozygous for HRT and susceptible to TCV.

Given our results with the genomic transformants, we anticipated that transgenic plants expressing HRT under the control of the powerful constitutive CaMV 35S cDNA promoter would predominantly exhibit extreme resistance to TCV. However, these lines exhibited only low HRT expression, similar to that seen in Di-17 plants. After TCV infection, these plants developed an HR and were susceptible to TCV. This finding suggests that very high expression of HRT may be deleterious to the plant and therefore is strongly selected against. HRT and other R proteins contain a large domain, termed NB-ARC by van der Biezen and Jones (1998), that includes the NBS and several other motifs that are shared with CED4 from Caenorhabditis elegans and its mammalian homolog APAF-1. CED4 and APAF-1 function as adapter molecules, tethering procaspases to the antiapoptotic factors CED9 and BCL2, respectively. This facilitates caspase activation by aggregation, which in turn leads to activation of a cell death program (Green, 1998; Raff, 1998). Perhaps HRT and other R proteins containing this motif can initiate a cell death program, even in the absence of their cognate Avr factors, if they are expressed in high amounts.

Interestingly, our HRT-overexpressing Col-0 lines differ from the Pto-overexpressing tomato lines described by Tang et al. (1999) in several important respects. Their 35S:Pto overexpressers developed a micro-HR and exhibited increased amounts of SA and expression of PR in the absence of infection. Moreover, their lines showed enhanced resistance to several different pathogens, reminiscent of systemic acquired resistance. In contrast, our HRT overexpressers did not develop a micro-HR in the absence of TCV infection, failed to express high amounts of PR-1 even after infection, and did not exhibit enhanced resistance to P. parasitica Emco5.

**The CP of TCV Is the Avirulence Determinant**

Oh et al. (1995) previously demonstrated that replacing the TCV CP with that of the related cardamine chlorotic fleck virus, which is virulent on Di-0 plants, produced a chimeric virus capable of systemically infecting Di-0 plants. Adding two amino acids to the N terminus of the TCV CP had no effect on CP stability or formation of virions but did allow systemic spread of the mutant virus in Di-0 plants (Wang and Simon, 1999). Moreover, generating mutations at the N terminus of the TCV CP produced hypervirulent strains of TCV that failed to elicit an HR and spread systemically in Di-17 plants (Y. Zhao, D. Dempsey, D.F. Klessig, and K. Wobbe, unpublished results). These results suggest that the TCV CP is the Avr factor. Confirming this possibility, we show that all of the CP-expressing F1 progeny from crosses between transgenic Col-0::TCV CP plants and either Di-17 or transgenic Col-0::HRT plants developed systemic necrosis and died (Figure 6). Death did not occur until ~2 days after initiation of the first leaf, suggesting that some component essential for the HR (possibly HRT) is developmentally regulated. This finding is consistent with the earlier discovery that cell death is developmentally regulated in tomato seedlings expressing the Cf-9 resistance gene and a transgene encoding the corresponding C. fulvum Avr9 protein (Hammond-Kosack et al., 1994; Honée et al., 1995). It also correlates with the demonstration that resistance to TCV in Arabidopsis and to Xanthomonas oryzae pv oryzae in rice increases with developmental age (Dempsey et al., 1993; Century et al., 1999).
With the identification of the TCV Avr factor, the cloning and characterization of HRT, and the identification of RRT, some of the framework required for elucidating the signaling pathway for TCV resistance is now available. Our results suggest that the amount of HRT expression and the presence of the RRT allele determine the resistance status of the plant. In Di-17 and Di-0 plants, which contain only the rrt allele, low HRT expression can confer both an HR and resistance (Figure 7A). In contrast, in the presence of the RRT allele (e.g., in the Col-0::HRT transgenic plants), low HRT expression confers HR development without viral resistance. Assuming that RRT suppresses HRT-mediated resistance, its presence appears to prevent the inhibition of TCV replication or spread but not the development of an HR (Figure 7B). However, the ability of RRT to suppress resistance can gradually be overcome by successively more expression of HRT (Figures 7C and 7D).

It has recently been suggested that extreme (HR-independent) resistance, resistance with a localized HR, and systemic HR are all variations in a continuum of responses that can be activated in the host plant by interaction between an Avr protein and an R protein (Bendahmane et al., 1999). In that model, the concentrations of the interacting components (e.g., Avr and R proteins, assuming that they interact directly), their time of appearance relative to the replication cycle of the pathogen, and the affinity of the components all play a role in determining the eventual response of the plant. A high affinity of the R protein for its cognate Avr determinant, as may be the case for Rx and the PVX CP, would allow the pathogen to be localized to the initially infected cell, thereby producing an HR-independent resistance. Because resistance in Di-17 and Di-0 plants is associated with an HR, the affinity of HRT for the TCV CP may be weaker. In this case, the pathogen would spread to the surrounding cells before resistance is fully activated. The infected cells would then undergo programmed cell death, leading to formation of necrotic lesion. In HRT-overexpressing transgenic Col-0 plants, however, highly increased amounts of HRT might

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**Figure 7. Model of the Interaction among HRT, RRT, and CP during the Course of TCV Infection.**

(A) The interaction of HRT and the TCV CP (directly or indirectly) initiates a signaling cascade(s) that induces development of both an HR and resistance, which blocks TCV replication and spread. A second gene, RRT, regulates resistance to TCV but does not influence HR development. The dominant RRT allele in Col-0 may act as a suppressor of HRT-mediated resistance. In this scenario, the rrt allele in Di-17 is nonfunctional, allowing resistance to develop.

(B) In transgenic Col-0 plants producing low amounts of HRT, the formation of a CP-HRT complex triggers an HR, but RRT suppresses development of HRT-mediated resistance, which results in susceptibility.

(C) In conjunction with the CP obtained by uncoating the virion, the high amount of HRT resulting from overexpression initiates a rapid and strong defense signal. This signal cannot be suppressed by RRT and is sufficiently rapid and strong to prevent TCV spread and replication beyond the initially infected cell. Therefore, too few cells are infected to develop a macro- or micronecrotic lesion.

(D) More modest overexpression of HRT leads to a signal of intermediate amplitude, which allows more cells to be infected and thereby forms a micro-HR. Moreover, this moderately strong signal is not always adequate to overcome the negative effect of RRT on the development of resistance; therefore, systemic spread of the virus occasionally occurs.
drive the equilibrium toward formation of an HRT-CP complex, thereby activating defense responses very early in the infection cycle and producing HR-independent resistance (Figure 7C). Those transgenic plants that express HRT moderately might activate resistance more slowly, allowing more cells to become infected and thereby producing a micro-HR (Figure 7D). In addition, moderate expression of HRT might not always be sufficient to overcome the negative effect of RRT on resistance, allowing systemic infection to occur. Why moderate HRT expression is sufficient to confer resistance in some transgenics and not in others is currently unclear. However, we have previously observed that variations in environmental conditions can affect resistance in Di-17 plants (Dempsey et al., 1993). Thus, multiple factors, including the presence of HRT and RRT, their extent of expression, and possibly various environmental conditions, appear to be involved in determining the response of Arabidopsis to TCV infection.

METHODS

Molecular Marker Construction

The cleaved amplified polymorphic sequence (CAPS) markers used with the resistant Dijon ecotype of Arabidopsis thaliana crossed with the susceptible Columbia ecotype (Di-17 × Col-0) mapping population were developed either by conversion of markers in Col-0 or from sequences generated from yeast artificial chromosome (YAC) ends. The marker DFR has been described elsewhere (Dempsey et al., 1997). A new polymorphism between Col-0 and Di-17 was discovered from the Col-0 marker agp6 by sequencing a Col-0 cDNA clone supplied by Andrea Prescott (John Innes Centre, Norwich, UK) and amplifying the corresponding fragments from Di-17 and Col-0 genomic DNA with primers 5'-TCTCAGCGAAGATAGGAGTGAAG-3' (agp forward [f]) and 5'-GTGTAACATATAACAGCGACAA-3' (agp reverse [r]), followed by digestion with MboI. Similarly, mi194 and mi83 (Liu et al., 1996) were shown to be polymorphic between Di-17 and Col-0 by using the appropriate pairs of primers 5'-AAATGT-TACCAGCTCGTGCCAATG-3' (mi194f) and 5'-TCCAGAGAAGAAGATAGGTGAAAC-3' (mi194r), and 5'-CAATCTTCA-AGCACC-3' (mi83f) and 5'-TCGAAGGAAATCTCTACAAATATA-3' (mi83r), followed by digestion with BsaAI and Ddel, respectively. 4RL was developed by inverse polymerase chain reaction (IPCR) from the left end of the Col-0 YAC CIC4E12 (Creusot et al., 1995) and using the enzyme Rsal. The sequence of the IPCR fragment allowed amplification of fragments from Col-0 and Di-17 with 5'-CAATCTTCA-TAAATCATACACTCCCTCGAT-3' (4RLf) and 5'-ACTAATTTGATATACCTTCTATG-3' (4RLr) primers. The 4RL marker is polymorphic with BsaAI.

Turnip Crinkle Virus Inoculation and Recombinant Screen

Turnip crinkle virus (TCV) inoculum was produced by in vitro transcription from the plasmid pT7TCV66 (Oh et al., 1995). Plant growth conditions and inoculation procedures were as previously described (Dempsey et al., 1993). A population of 1223 F2 plants (Col-0 × Di-17) was screened for recombination between the CAPS markers DFR and mi83. The genotype at the HRT locus was determined by TCV inoculation of 16 to 20 F2 plants from selected recombinant F2 individuals. Plants were scored for HR at 3 days postinoculation (DPI). The genotype of the F2 parent was assumed to be homozygous hrt or HRT if no plants or if all the plants had an HR, respectively. Otherwise, the genotype was assumed to be heterozygous HRT/hrt. Two recombinants further constrained HRT to an 0.8-cM region between the internal markers mi194 and 4RL. Based on the YAC contig in the DFR region in the Col-0 ecotype (Schmidt et al., 1997), the region between mi194 and 4RL was presumed to be ~220 kb. Construction of a BAC contig from Di-17 later confirmed this estimation (see below).

BAC Library Construction and Screening

Using the procedure described by Zhang et al. (1995), high molecular mass genomic DNA was isolated from Di-17 plants, embedded in agarose microbeads, partially digested with HindIII, and size-fractionated by pulse field gel electrophoresis. Size-selected DNA was then ligated to a HindIII-digested pBeloBACII plasmid vector and electroporated into Electromax DH10B cells (Gibco BRL, Rockville, MD). The library consisted of 6144 clones, and the insert size ranged from 50 to 150 kb (with an average of 90 kb). The library was arrayed onto four Hybond N+ membranes (Amersham Pharmacia, Piscataway, NJ), as described by Woo et al. (1994), and hybridized with DNA probes developed from markers mi194, agp6, or 4RL under standard high-stringency hybridization conditions as defined below. The ends of the bacterial artificial chromosome (BAC) clones were sequenced directly, and a contig of four overlapping BAC clones spanning the markers mi194 and 4RL was assembled by cross-hybridization of BAC clones to DNA probes generated from BAC end sequences.

Plasmids

pBIBAC2 is a 25-kb binary plasmid based on the F1 and R1 origins of replication (Hamilton et al., 1996) and contains a unique BamHI cloning site in the gene SacB, which confers sensitivity to sucrose in Gram-negative bacteria. Each recombinant pBeloBACII plasmid was partially digested with BamHI, and 30- to 40-kb fragments were subcloned into pBIBAC2 by standard techniques (Ausubel et al., 1987). In some cases, the cloned fragments in BIBAC2 also contained the 7-kb pBeloBACII vector. The size of the inserts was determined by using field inversion gel electrophoresis (Bio-Rad, Hercules, CA) after digestion of the plasmids with NotI (the BamHI site in pBIBAC2 is flanked by two NotI sites). The order of the subclones relative to the BAC contig was determined by polymerase chain reaction (PCR) amplification with primers from the CAPS markers or from primers produced by sequencing the BAC ends. The order and orientation of the BIBAC2 subclones were confirmed by high-stringency DNA gel blot analysis (Ausubel et al., 1987). High-stringency DNA gel blot hybridization, used throughout, is defined as a final wash of 15 min in 0.1× SSC (1× SSC is 150 mM sodium chloride and 15 mM sodium citrate) and 0.1% SDS at 65°C.

TCV coat protein (CP) transgenic plants were constructed by amplying the CP gene from the TCV cDNA in pT7TCV66 by using the enzyme rTth DNA polymerase-XL (Perkin-Elmer, Norwalk, CT) while incorporating Smal and BamHI restriction sites at the 5’ and 3’ ends.
of the open reading frame (ORF), respectively. The fragment was cloned into the pGEM-T Easy vector (Promega, Madison, WI), sequenced, and then recloned as a Smal-BamHI fragment into pDH51 (Pietrzak et al., 1986) between the 35S promoter and polyadenylation site. The CP gene, including the cauliflower mosaic virus (CaMV) 35S promoter and polyadenylation site, was then transferred from pDH51 into the binary vector pBIN19 (Bevan, 1984) as an EcoRI fragment.

Screening and Cloning HRT cDNA

A cDNA library, made in the λZAP Express vector (Stratagene, La Jolla, CA) from a mixture (1:1:1:2 [w/w]) of mRNA from leaves harvested at 6, 12, 19, and 24 hr, respectively, after TCV infection of Di-17 plants, was screened with the E2 fragment, which conferred an HR to TCV-inoculated Col-0 (Ausubel et al., 1987). Of the 16 cDNAs recovered, 13 cross-hybridized and had identical 3’ ends, as determined by partial sequencing. Their 5’ ends were found to be homologous to different regions of RPP8. cDNA clone H116, which contained the entire ORF, was inserted behind the 35S promoter in the Smal site of pDH51 as a Klenow fragment of DNA polymerase I-blunted EcoRI-NotI fragment. The cDNA with the 35S promoter and polyadenylation site was cut from pDH51 as an EcoRI fragment and was cloned into pBIN19 for transformation into plants.

Transformation of Arabidopsis

Subclones of BAC genomic DNA, cDNA, or the TCV CP were transformed into either GV3101 or C58C1 Agrobacterium by electroporation (Cooley et al., 1991). Arabidopsis plants were transformed using the vacuum infiltration technique (Bechtold, 1998), as modified by A. Bendalmane and D. Baulcombe (personal communication). Transgenic plants were detected as seedlings growing on 0.5 × Murashige and Skoog salts (Murashige and Skoog, 1962) plus 0.8% agar and 50 μg/ml kanamycin. The genotype of the HRT transgenic plants was confirmed using a polymorphism specific to HRT to TCV-inoculated Col-0 (Ausubel et al., 1987). Of the 16 cDNAs recovered, 13 cross-hybridized and had identical 3’ ends, as determined by partial sequencing. Their 5’ ends were found to be homologous to different regions of RPP8. cDNA clone H116, which contained the entire ORF, was inserted behind the 35S promoter in the Smal site of pDH51 as a Klenow fragment of DNA polymerase I-blunted EcoRI-NotI fragment. The cDNA with the 35S promoter and polyadenylation site was cut from pDH51 as an EcoRI fragment and was cloned into pBIN19 for transformation into plants.

Reverse Transcription–PCR

Total RNA was isolated with Trizol reagent (Gibco BRL), using methods supplied by the manufacturer. RNA quality and concentration were determined by gel electrophoresis and determination of A₂₆₀. Reverse transcription (RT)-PCR was performed with Ready-To-Go RT-PCR Beads (Amersham Pharmacia). Essentially, 2 μg of total RNA was reverse transcribed in a 50-μL reaction volume with RT-specific primers (5’-CGTATCCAAAGCTCTTCTCTTG-3’ and 5’-AATGCAGAGTTTGGATACAG-3’) for 30 min at 42°C. The reverse transcriptase was heat-inactivated at 95°C for 5 min, cooled to 4°C, and divided into five 10-μL aliquots. To each aliquot was added 1 μL of a solution containing 40 μg of RNase A and an appropriate amount (54 to 0.2 pg) of the comparator plasmid (see below). The cDNA was amplified for 15 cycles consisting of 1 min at 95°C, 1 min at 55°C, and 1 min at 72°C, followed by 5 min at 72°C. The amplified cDNA was subjected to electrophoresis (in 1.2% agarose and TAE buffer [40 mM Tris-acetate and 1 mM EDTA]), blotted by alkaline transfer (Ausubel et al., 1987), and hybridized at high stringency to 32P-labeled H116 cDNA. The amount of hybridization to the amplified HRT cDNA and to the comparator was quantified with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

The comparator plasmid is used at known concentrations as an internal PCR standard. The number of cycles was decreased to 15 to ensure that amplification of the comparator did not compete with the wild-type HRT template (Martorana et al., 1999). The comparator plasmid was constructed from the HRT cDNA fragment. The HRT full-length cDNA (H116) in pBK-CMV (Stratagene) was digested at a unique PstI site in the HRT ORF and religated in the presence of an EcoRI linker. In addition to the linkage, the ligation resulted in an insertion of 150 bp of extraneous Escherichia coli DNA. The concentration of the comparator plasmid was determined by both A₂₆₀ and comparison with known standards on an agarose gel after electrophoresis and staining with ethidium bromide. Known amounts of the comparator were added to the RT-PCR amplifications at threefold dilutions. By comparing the amount of amplified HRT with that of the comparator molecule, the amount (in picograms) of starting HRT cDNA template was estimated. Each quantification was performed in triplicate.

Peronospora parasitica Inoculation and Scoring

P. parasitica isolate Emco5 inoculum was prepared from infected, frozen (~80°C) leaf tissue by vortex-mixing in 0°C water and used immediately to inoculate cotyledons of the susceptible ecotype Wassilewskija. Plants were incubated at 17°C and >90% relative humidity, with an 8-hr-light (5000 Lux) and 16-hr-dark cycle before and after inoculation. Profuse sporulation was seen after 5 days. The spores were harvested by excising the infected tissue (5 to 9 DPI) and vortex-mixing in 0°C sterile water. The spore concentration, determined with a hemocytometer, was 10⁷ to 10⁸ per mL. The spore suspension was used immediately to inoculate test plants by spraying or applying one drop per leaf on two or three leaves per plant. The plants were incubated as above, and the number of sporangia-phores per leaf was determined 5 DPI with a dissecting microscope.

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Members of the Arabidopsis HRT/RPP8 Family of Resistance Genes Confer Resistance to Both Viral and Oomycete Pathogens
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