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Arabidopsis *RAR1* Exerts Rate-Limiting Control of *R* Gene–Mediated Defenses against Multiple Pathogens

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We have identified the Arabidopsis ortholog of barley *RAR1* as a component of resistance specified by multiple nucleotide binding/Leu-rich repeat resistance (*R*) genes recognizing different bacterial and oomycete pathogen isolates. Characterization of partially and fully defective *rar1* mutations revealed that wild-type *RAR1* acts as a rate-limiting regulator of early *R* gene–triggered defenses, determining the extent of pathogen containment, hypersensitive plant cell death, and an oxidative burst at primary infection sites. We conclude that *RAR1* defense signaling function is conserved between plant species that are separated evolutionarily by 150 million years. *RAR1* encodes a protein with two zinc binding (CHORD) domains that are highly conserved across eukaryotic phyla, and the single nematode CHORD-containing homolog, *Chp*, was found previously to be essential for embryo viability. An absence of obvious developmental defects in null Arabidopsis *rar1* mutants favors the notion that, in contrast, *RAR1* does not play a fundamental role in plant development.

INTRODUCTION

In countering attack by microbial pathogens or insects, plants have evolved resistance (*R*) genes that specifically recognize corresponding pathogen avirulence (*avr*) genes to trigger plant defenses (Dangl and Jones, 2001). Two plant *R* gene–encoded proteins, tomato Pto and rice Pi-ta, have been shown to interact physically with their pathogen Avr counterparts, AvrPto and Avr-Pita, respectively, in in vitro assays (Scofield et al., 1996; Tang et al., 1996; Jia et al., 2000). Other plant R proteins may associate with pathogen Avr proteins indirectly within a protein complex (Leister and Katagiri, 2000). In the absence of a corresponding *R* gene, the pathogen is able to colonize its host. Some Avr proteins are virulence factors that facilitate pathogen growth or interfere with basal plant defenses (Nimchuk et al., 2000; Staskawicz et al., 2001). R-Avr protein recognition commonly involves localized programmed plant cell death (the hypersensitive response [HR]), an oxidative burst producing reactive oxygen intermediates (ROI), and the accumulation of salicylic acid (SA), a phenolic molecule necessary for the induction of systemic immunity (systemic acquired resistance) (Feys and Parker, 2000).

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Plant R proteins share a limited repertoire of motifs with animal proteins that control innate immunity (Staskawicz et al., 2001). The most prevalent *R* gene class encodes predicted cytosolic proteins with a central nucleotide binding (NB) domain and C-terminal Leu-rich repeats (LRRs) (Dangl and Jones, 2001). At least one NB-LRR–type protein, Arabidopsis RPM1, is tethered to the plasma membrane, where it may encounter bacterial Avr proteins that are secreted into the plant cell (Boyes et al., 1998; Nimchuk et al., 2000). NB-LRR proteins fall into two subclasses based on their different N-terminal motifs. One group possesses an N-terminal coiled-coil (CC) domain. The second group has N-terminal similarity to the cytoplasmic Toll Interleukin-1 Receptor (TIR) domains of human and *Drosophila* Toll-like receptors (Dangl and Jones, 2001).

Mutational analyses in plants have led to the identification of genes that are essential for the full expression of *R* gene–specified resistance, providing an important first step in the elucidation of defense signaling (Feys and Parker, 2000). In Arabidopsis, *EDS1* and *PAD4*, which encode lipase-like proteins (Falk et al., 1999; Jirage et al., 1999), are necessary for resistance conferred by TIR-NB-LRR genes (Aarts et al., 1998; Feys et al., 2001). NDR1, a potentially membrane-associated protein (Century et al., 1997), is dispensable for this resistance but is essential for the function of most, but not all, CC-NB-LRR proteins (Aarts et al., 1998; McDowell et al., 2000). Thus, EDS1/PAD4 and NDR1 appear to specify

distinct processes that are required by different R protein structural types.

Another resistance signaling gene, *RAR1*, was identified in mutational screens for suppressors of *Mla12* resistance in barley to the powdery mildew fungus *Blumeria* (*Erysiphe*) *graminis* f.sp. *hordei* (Freialdenhoven et al., 1994). *RAR1* is required by multiple barley *Mla* genes as well as other unlinked powdery mildew resistance loci (Jørgensen, 1996; Halterman et al., 2001; Zhou et al., 2001). Barley *rar1* mutant plants are impaired in whole cell ROI accumulation and in the HR of attacked host epidermal cells in *Mla12*-specified resistance, suggesting that RAR1 acts early in the plant resistance signaling cascade (Freialdenhoven et al., 1994; Shirasu et al., 1999). *RAR1* encodes a 25-kD, putatively cytosolic protein containing two 60–amino acid Cys- and His-rich (CHORD) Zn^{2+} binding domains that are conserved in sequence and tandem organization in all eukaryotic phyla examined (Shirasu et al., 1999). Plant RAR1 proteins possess an additional 20–amino acid motif with three invariant Cys residues and a His (denoted the CCCH domain) between CHORD domains I and II that is absent from related nonplant sequences. RNA interference of the single CHORD-containing gene in *Caenorhabditis elegans*, *Chp*, caused defects in germline development and embryo lethality (Shirasu et al., 1999), suggesting that *Chp* has a fundamental role in worm development.

It is unresolved whether the molecular functions of plant RAR1 proteins are conserved and whether these are shared with CHORD-containing homologs in metazoans. It was speculated that the lack of copy number expansion of CHORD proteins and the strict tandem organization of CHORD domains I and II across plant and animal phyla may indicate a conserved unit of function (Shirasu et al., 1999). Alternatively, unknown common biochemical function(s) of CHORD units may have been recruited for different biological processes in different species. The lack of definitive null *rar1* mutations in barley left open the possibility that a fundamental cellular role of RAR1 may exist in plants that is fulfilled by residual functional protein in the two mutant alleles identified, *rar1-1* and *rar1-2*.

Our mutational screens for genetic suppressors of *RPP5* resistance in Arabidopsis accession Landsberg *erecta* (L*er*) to the downy mildew pathogen *Peronospora parasitica* have identified a series of partially and fully defective mutations within the single *RAR1* ortholog. We show that *RAR1* is an early component of *R* gene–triggered resistance against avirulent Peronospora and *Pseudomonas syringae* pv *tomato* isolates, exerting rate-limiting control of defense signal fluxes leading to hypersensitive plant cell death. We also find that *RAR1* is used by both TIR- and CC-NB-LRR proteins, indicating that its recruitment is not conditioned by a particular R protein structural type. Isolation of definitive null *rar1* mutations supports the notion that *RAR1* in plants has evolved a signaling capability essential for plant defense against pathogens but dispensable in fundamental processes of plant development.

RESULTS

Isolation of Multiple *rar1* Mutants

Expression of the *RPP5* resistance gene in accession L*er* confers resistance to Peronospora isolate Noco2 (Parker et al., 1997). Infection with Noco2 causes restriction of the pathogen to inoculation sites and an associated HR. L*er rpp5* mutants or the susceptible accession Columbia (Col-0) do not display an HR in response to Noco2. Instead, the pathogen grows beyond inoculation sites to colonize the plant systemically, resulting in asexual sporulation on cotyledons and leaves 5 to 7 days after infection. Mutational screens for suppressors of *RPP5* resistance were performed on ~220,000 M2 seedlings derived from fast neutron (FN)– or ethyl methanesulfonate (EMS)–mutagenized L*er* seed. Susceptible mutants, which show substantial levels of pathogen sporulation, and partially susceptible mutants, which permit only low sporulation levels, were isolated.

Crosses between each of the newly identified mutant lines and wild-type L*er* produced resistant F1 plants, consistent with all of the mutations being recessive or semidominant. Allelism tests were performed between the mutant lines and with the previously isolated *rpp5*, *eds1*, and *pad4* mutants (Parker et al., 1997; Falk et al., 1999; Jirage et al., 1999). These analyses revealed two new complementation groups, designated *rpr1* and *rpr2* (required for *RPP5* resistance; Parker et al., 2000). Here, we present our characterization of the *rpr2* complementation group, comprising one FN- and five EMS-generated alleles. A comparison of the six isolated *rpr2* alleles revealed distinct susceptible infection phenotypes, three showing substantial pathogen sporulation and three (all EMS mutants) showing sparse sporulation (Table 1). F2 seedlings generated from crosses between each of the six independent *rpr2* alleles and L*er* segregated in a ratio of 3:1 (resistant:susceptible plants; data not shown), indicating that *rpr2* is a single recessive mutation.

A map-based cloning strategy was used to isolate the *RPR2* gene, and its location was narrowed to a 220-kb region on the lower arm of chromosome 5 (see Methods). Within this region, we identified a gene with high sequence similarity to barley *RAR1* (Shirasu et al., 1999). Therefore, we determined whether *rpr2* contained mutations in Arabidopsis *RAR1* by amplification by polymerase chain reaction and sequencing of genomic DNA from the FN-derived *rpr2-1* line and wild-type L*er*. Alignment of the *RAR1* sequences from *rpr2-1* and L*er* revealed a 5-bp deletion in *rpr2-1* (Figure 1A). *RAR1* DNA was sequenced in the remaining five *rpr2* alleles, and each was found to have a mutation within the *RAR1* gene (Figures 1A and 1B). We concluded that *RPR2* corresponds to the Arabidopsis ortholog of barley *RAR1*. Our FN *rpr2-1* allele is denoted *rar1-10*, and the five EMS alleles are denoted, consecutively, *rar1-11* to *rar1-15*, in a convention that accords with that used by J. Dangl, K. Shirasu, and colleagues to distinguish *rar1* mutations identified in different Arabidopsis accessions (see Methods).

Table 1. Summary of *rar1* Mutations in L*er*

Allele	Mutagenesis	Class[a]	Nucleotide Change[b]	Amino Acid Change	Defect	Predicted Size (Amino Acids)
rar1-10	FN	S	5-bp deletion (561 to 565 bp)	Multiple	Frameshift and premature stop	212
rar1-11	EMS	S	C to T (154 bp)	Q to STOP	Premature stop	51
rar1-12	EMS	PS	G to A (581 bp)	W to STOP	Premature stop	193
rar1-13	EMS	S	G to A (3′ splice site in intron 3)	Unknown	Splice site defect	Unknown
rar1-14	EMS	PS	G to A (508 bp)	E to K	Amino acid substitution	226
rar1-15	EMS	PS	G to A (590 bp)	C to Y	Amino acid substitution	226

[a] PS, partially susceptible; S, susceptible.
[b] The position of the nucleotide change corresponds to the *Rar1* coding sequence (see Figure 1A).

The mutations found in *rar1-10* to *rar1-15* are summarized in Table 1. Two of the three mutants showing substantial sporulation contain premature stop codons. *rar1-11* has a stop codon at the beginning of the CHORD I domain, whereas *rar1-10* (after a frameshift) possesses a stop codon within CHORD II (Figure 1A). In *rar1-13*, the G nucleotide of the 3′ splice site consensus of intron 3 is mutated (Figure 1B). This consensus sequence is essential for correct processing of primary mRNA transcripts in plants and higher eukaryotes (Goodall and Filipowicz, 1991). Of the mutants classed as partially susceptible, *rar1-12* has a stop codon, and *rar1-14* and *rar1-15* have amino acid substitutions within CHORD II. In *rar1-15*, a Cys residue (Cys-197) that is invariant in all known plant and animal CHORD domains (Shirasu et al., 1999) is substituted by Tyr. In *rar1-14*, Glu-170 (acidic) is changed to Lys (basic). Although this residue is not conserved strictly in CHORD I or II, an acidic residue is found invariably at this position in all plant CHORD domains examined.

Expressed sequence tag and genomic DNA database searches show *RAR1* to be a probable single-copy gene in all plant species analyzed. We confirmed this in accessions L*er* and Col-0 by DNA gel blot analysis (data not shown). The high level of similarity between Arabidopsis and barley *RAR1* is shown in Figure 1C. Particularly striking is the almost complete conservation of their CHORD I, CHORD II, and CCCH domains.

Characterization of *rar1* Mutant Defects

The *rar1* mutant lines were backcrossed at least once to parental L*er* for phenotypic analysis. We determined the extent of disease susceptibility in the different *rar1* mutants by measuring numbers of conidiospores on leaves 7 days after inoculation of plants with Noco2. As shown in Figure 2, sporulation on all *rar1* mutant lines was significantly lower than on *rpp5* or *eds1*, which displayed susceptibility and supersusceptibility, respectively (Parker et al., 1997; Feys et al., 2001). Three mutants, *rar1-10*, *rar1-11*, and *rar1-13*, each exhibited ~50% of the spore levels counted on *rpp5* plants. Thus, even the strongest *rar1* mutants (see below) do not completely disable *RPP5* resistance. Our classification of *rar1-12*, *rar1-14*, and *rar1-15* as partially susceptible mutants (Table 1) was consistent with lower spore counts on these plants compared with *rar1-10*, *rar1-11*, and *rar1-13*.

We examined the effects of the different *rar1* mutations on *RAR1* transcript and RAR1 protein abundance in healthy (pathogen-unchallenged) tissues of mutant and wild-type plants. As shown in Figure 3A, *RAR1* mRNA levels in *rar1-10*, *rar1-12*, *rar1-14*, and *rar1-15* were similar to those measured in wild-type L*er*. In contrast, *RAR1* transcripts were undetectable in *rar1-11* and were depleted severely in *rar1-13*. We presume that early protein truncation in *rar1-11* and the intron 3 splice defects in *rar1-13* (Table 1) led to transcript instability. RAR1 protein amounts in the *rar1* mutant lines were determined by probing blots of soluble protein extracts with polyclonal antisera raised against full-length RAR1 protein. Anti-RAR1 antisera detected a single ~30-kD band in wild-type protein extracts (Figure 3B). RAR1 was not detectable in either *rar1-11* (consistent with the transcript analysis) or *rar1-13*. We deduced from these data that *rar1-11* and *rar1-13* are complete loss-of-function mutations.

Truncated RAR1 proteins were observed in *rar1-10* and *rar1-12* at sizes consistent with those predicted by the positions of their premature stop codons (Figure 3B, Table 1). However, their levels were much lower than those in wild-type RAR1, presumably as a result of the reduced stability of the truncated products. Because *rar1-10* displayed a level of Noco2 sporulation equivalent to that of the null *rar1* alleles, we concluded that *rar1-10* is most likely also a null mutant. There was low residual activity associated with the truncated RAR1 protein in *rar1-12*, reflected by significantly lower Noco2 sporulation levels than on *rar1-10*, *rar1-11*, or *rar1-13* (Figure 2). Although the E170K CHORD II mutation in *rar1-14* did not affect RAR1 protein abundance, the C197Y CHORD II mutation in *rar1-15* resulted in a reduction of protein to approximately half the level of the wild type (Figure 3B).

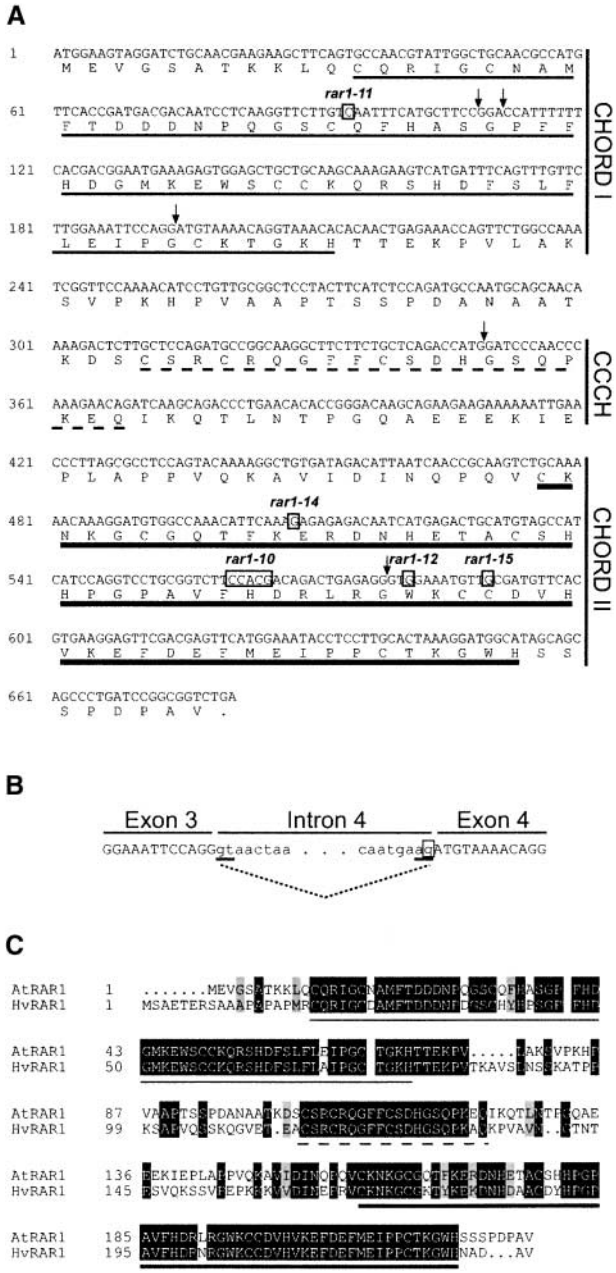

Figure 1. Sequence Analysis of Wild-Type and Mutant Alleles of Arabidopsis *RAR1*.

(A) Coding region and deduced amino acid sequence of *RAR1* in accession L*er*. Arrows mark the positions of introns 1 to 5. The CHORD I, CHORD II, and CCCH domains are indicated by thin, thick, and broken underlines, respectively. Nucleotide changes in *rar1-10*, *rar1-11*, *rar1-12*, *rar1-14*, and *rar1-15* are boxed.

(B) Scheme of the intron 3 splice site defect in *rar1-13*. The sequence at the exon 3–intron 3–exon 4 boundary is shown. Intron sequences are displayed in lowercase letters, and exon sequences are displayed in uppercase letters. The 5′ and 3′ splice sites of intron 3 are underlined, and splicing of the wild-type RNA is shown with a dotted line. The nucleotide change in *rar1-13* is boxed.

(C) Amino acid sequence alignment of Arabidopsis (At) and barley (Hv) RAR1. The proteins have 60% identity. Identical and similar residues are displayed in black and gray boxes, respectively. The CHORD I, CHORD II, and CCCH domains are underlined as in **(A)**.

Pathogen-Triggered Cell Death Responses of *rar1* Plants

The susceptibility of barley *rar1* mutants to powdery mildew was shown to coincide with a failure in epidermal host cell death and ROI accumulation (Freialdenhoven et al., 1994; Shirasu et al., 1999). We investigated whether similar defects were apparent in Arabidopsis *rar1* mutant responses to Peronospora. For this analysis, we selected the null mutant *rar1-11* and a partially defective mutant, *rar1-15*, and compared responses with those of L*er* (*RPP5*) and *rpp5* mutant plants.

Lactophenol trypan blue staining was used to monitor pathogen growth and plant cell death at various times after inoculation with Noco2 (Rustérucci et al., 2001). As shown in Figure 4A, L*er* developed discrete HR lesions on inoculated leaves. Noco2 mycelium did not extend beyond these interaction sites. In contrast, *rpp5* mutants permitted Noco2 growth in the absence of plant cell death. At early time points (2 days after inoculation; Figure 4A), the majority (>95%) of interaction sites in null *rar1-11* mutant leaves resembled those in *rpp5* leaves. A small proportion (<5%) of sites exhibited an attenuated HR, and at later times, trails of collapsed mesophyll cells were found to be associated with the growing mycelium (Figure 4A).

Thus, complete loss of *RAR1* function in *rar1-11* seemed to cause a strongly delayed HR resulting in trailing plant cell necrosis, which was borne out by the appearance at late time points (5 days after inoculation) of autofluorescing dead cells on leaves of *rar1-11* but not *rpp5* plants (Figure 4B). In the partially defective *rar1-15* plants, an attenuated HR was observed at most plant–pathogen interaction sites, and Noco2 mycelium was seen to emerge from these areas (Figure 4A). At later times, there was less extensive pathogen growth and more extreme trailing plant cell death than was observed in *rar1-11* (Figures 4A and 4B).

As a measure of ROI accumulation, we used 3,3-diaminobenzidine polymerization (Thordal-Christensen et al., 1997; Rustérucci et al., 2001) to determine if the *rar1* null and partial mutants were compromised in *RPP5*-triggered H_2O_2 generation upon Noco2 challenge. In L*er* leaves, whole cell H_2O_2 was detected at the same early time point (2 days after inoculation) as an HR and remained restricted to these discrete areas of dead or dying cells over a 5-day time course (Figure 4C). No whole cell ROI accumulation was observed at interaction sites of *rpp5* plants or in the majority of sites in *rar1-11* plants (Figure 4C). In contrast, ROI accumulation was readily detectable in most pathogen inoculation sites of *rar1-15*. At later times, the pattern of whole cell ROI

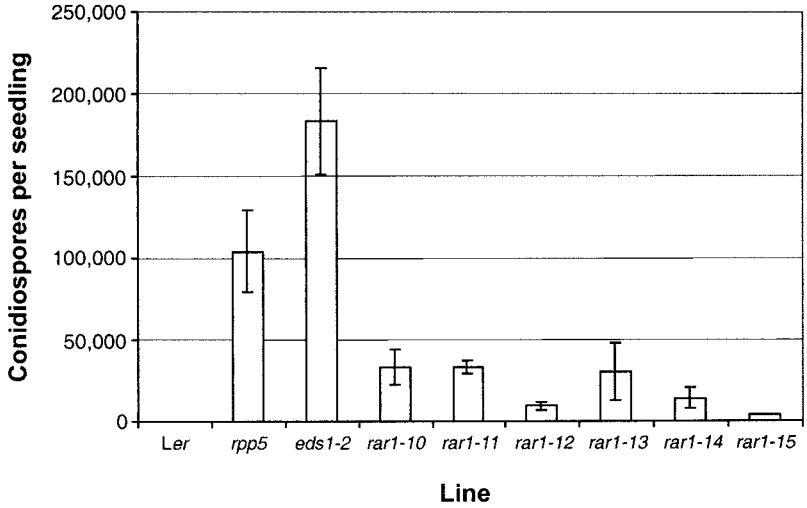

Figure 2. Asexual Sporulation of Peronospora Noco2 on Wild-Type and Mutant Plants.

Production of conidiospores on L*er*, *rpp5*, *eds1-2*, and six independent *rar1* mutants (*rar1-11* to *rar1-15*) was determined 7 days after inoculation. The data and standard error values shown are from three replicate samples per line in a single experiment. Similar results were observed in an independent experiment.

generation followed that of trailing plant cell death in *rar1-11* and *rar1-15*. We concluded from these data that *RAR1* acts as a rate-limiting component of early *RPP5*-conditioned defenses, operating upstream of hypersensitive plant cell death and its accompanying oxidative burst.

Analysis of Different *R* Gene Requirements for *RAR1*

We examined the requirements of different Arabidopsis *R* genes for *RAR1* by challenging wild-type and mutant plants with avirulent Peronospora or Pseudomonas isolates that are recognized by particular *R* genes (Table 2). Besides testing the responses of *R* genes present in L*er*, several *R* genes not found in L*er* but expressed in accessions Col-0 and Wassileskija were included. For the latter tests, the wild-type accessions were crossed with either *rar1-10* or *rar1-11*, and F2 generation plants were selected that were homozygous for wild-type or mutant *rar1* and that segregated for the respective *R* gene (Table 2; see Methods).

We found that *RAR1* was essential for resistance conferred by *RPP4*, a recognitionally distinct Col-0 ortholog of *RPP5* (van der Biezen et al., 2002). RAR1 also was required for full expression of resistance mediated by *RPP21* (a locus not yet defined) but was dispensable for the function of other *RPP* genes examined (Table 2). All four *R* genes (*RPM1*, *RPS2*, *RPS4*, and *RPS5*) tested against corresponding avirulent Pseudomonas isolates (expressing *avrRpm1*, *avrRpt2*, *avrRps4*, and *avrPphB*, respectively) required *RAR1* (Table 2). Figure 5A shows that the growth of Pseudomonas DC3000 harboring *avrRpm1*, *avrRpt2*, or *avrRps4* was similar in *rar1-11* leaves to that of virulent DC3000 (containing an empty vector) in either *rar1-11* or L*er*. Although *rar1-11* exhibited full susceptibility to these avirulent bacteria, it did not display a supersusceptibility phenotype that is characteristic of *eds1-2* mutants to either DC3000 expressing *avrRps4* or DC3000 alone (Figure 5A).

We found in all cases that disease symptom development correlated with bacterial growth in the different plant lines, as shown for plants inoculated with DC3000 expressing either *avrRpm1* or *avrRps4* (Figure 5B). In these assays, a distinction could be made between the susceptibility of *rar1-11* and the enhanced susceptibility of *eds1-2* to DC3000/*avrRps4*. In an accompanying article, Tornero et al. (2002) report a much lower dependence of *RPS4* on *RAR1* in accession Col-0 than we found for L*er RPS4*. We assessed whether *rar1-15*, which was partially defective in *RPP5* resistance (Figure 2), also suppressed bacterial resistance more weakly than the null *rar1-11* allele. The growth of all of the avirulent Pseudomonas strains was intermediate between that observed in *rar1-11* and resistant L*er* (Figure 5A).

These results demonstrate that *RAR1*, as in *RPP5* resistance, exerts a rate-limiting control of plant defenses to avirulent bacteria. In *rar1-15*, the timing and intensity of an *RPM1*-induced HR (Figure 5C) was not obviously different from that in wild-type plants after infiltration of concentrated suspensions of DC3000/*avrRpm1*, whereas HR (Figure 5C) were strongly attenuated in *rar1-11*. Expression of an *RPS2*-conditioned HR was not observed in leaves of either *rar1-11* or *rar1-15* (data not shown). We were unable to observe consistent HR development in wild-type or mutant plants after challenge with DC3000/*avrRPS4*.

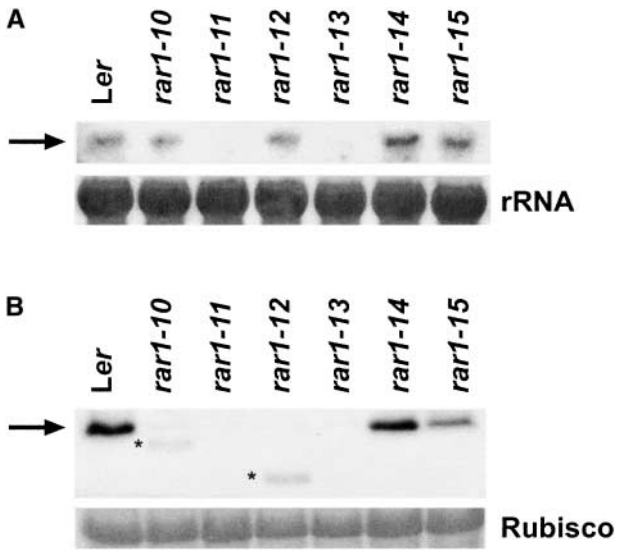

Figure 3. Molecular Characterization of the *rar1* Mutants.

(A) RNA gel blot analysis of *RAR1* transcript levels. Total RNA (20 μg) from 4-week-old healthy L*er* and *rar1* mutant seedlings was probed with a genomic *RAR1* DNA fragment. Equal loading was determined by visualization of rRNA on the filter with methylene blue.
(B) Immunoblot analysis of RAR1 protein. Total soluble protein extracts (50 μg) prepared from the same material used for the RNA analysis shown in **(A)** were probed with polyclonal anti-RAR1 antisera. Asterisks indicate truncated products detected in *rar1-10* and *rar1-12*. Equal loading was determined by Ponceau S staining of the filter. Similar results were obtained in a second independent experiment.

Defense-Related Gene Expression in *rar1*

Our analyses of *rar1* responses to Peronospora or Pseudomonas revealed deficiencies in defenses conditioned by certain *R* genes but no relaxation of resistance to virulent pathogens (Figures 2 and 5). We wanted to explore whether *RAR1* function is restricted to plants undergoing an *R* gene–triggered HR. SA accumulation is required for the expression of basal resistance and systemic immune responses to virulent pathogens in the absence of an HR (Gaffney et al., 1993; Cao et al., 1994; Reuber et al., 1998; Feys et al., 2001). Therefore, we compared induction profiles of the SA-responsive gene *PR1* in wild-type (L*er*) and *rar1-10* null mutant leaves after inoculation with avirulent (DC3000/*avrRpm1*) or virulent (DC3000) bacteria. We found that *PR1* expression was induced more rapidly in wild-type plants responding to DC3000/*avrRpm1* than to DC3000 alone over a 72-hr period (Figure 6). In *rar1-10* leaves, *PR1* induction was reduced strongly compared with that in wild-type leaves after inoculation with avirulent DC3000/*avrRpm1*, but it was similar to the wild-type response after inoculation with virulent DC3000 (Figure 6). Therefore, defects in *RAR1* appear not to be registered by plants in basal resistance.

DISCUSSION

RAR1 Function Is Conserved between Barley and Arabidopsis

We have identified the single Arabidopsis *RAR1* ortholog as an essential component of resistance conferred by NB-LRR–type *R* genes recognizing avirulent bacterial and oomycete pathogens. In *RPP5* resistance against Peronospora (Figure 4) and in *RPM1* resistance against Pseudomonas (Figure 5), *RAR1* functions at an early stage of plant defense, controlling expression of the HR and whole cell ROI generation at plant–pathogen interaction sites. In barley, *RAR1* operates upstream of epidermal cell ROI accumulation and the HR in *Mla12*-conditioned resistance to an ascomycete fungus (Shirasu et al., 1999). We conclude that the function of *RAR1* in disease resistance is conserved between monocotyledonous and dicotyledonous plant species that are separated evolutionarily by ~150 million years.

RAR1 Is Required by TIR- and CC-NB-LRR Resistance Genes

An important finding from this study is the identification of *RAR1*-dependent and *RAR1*-independent *R* genes in both the TIR- and CC-NB-LRR classes (Table 2). Thus, genetic recruitment of *RAR1* is not determined by a particular NB-LRR structural type, in contrast with the observed signaling preferences of TIR-NB-LRR–type genes for *EDS1* and *PAD4* and the majority of CC-NB-LRR genes for *NDR1* (Aarts et al., 1998; McDowell et al., 2000; Peart et al., 2002). This distinction raises questions about the mechanism(s) that control *RAR1* participation in the resistance pathway. We think it unlikely that the speed or intensity with which certain *R* genes induce an HR influence their *RAR1* signaling mode, because *RPM1*, which conditions a rapid and strong HR and efficient pathogen containment, had a *RAR1* requirement equivalent to that of *RPS4*, which triggers a weaker HR and restricts pathogen growth less effectively (Figure 5) (Rustérucci et al., 2001). Moreover, barley *Mla1* and *Mla6* specify temporally and spatially similar rapid epidermal cellular defenses against powdery mildew infection yet differ strikingly in their *RAR1* dependence (Zhou et al., 2001; Halterman et al., 2001).

It is significant that the *Mla1*- and *Mla6*-encoded CC-NB-LRR proteins are 92% identical, implying that other discriminatory mechanisms regulate the engagement of *RAR1* (see below). Our results suggest that *RAR1* activity is restricted to *R-avr* gene–triggered responses and do not extend to its involvement in basal resistance against virulent pathogens. Thus, the susceptibility of *rar1* to DC3000 was equivalent to that of wild-type plants and was not as extreme as in *eds1* plants exhibiting hypersusceptibility to this bacterial strain (Figure 5). Consistent with the bacterial growth data, the in-

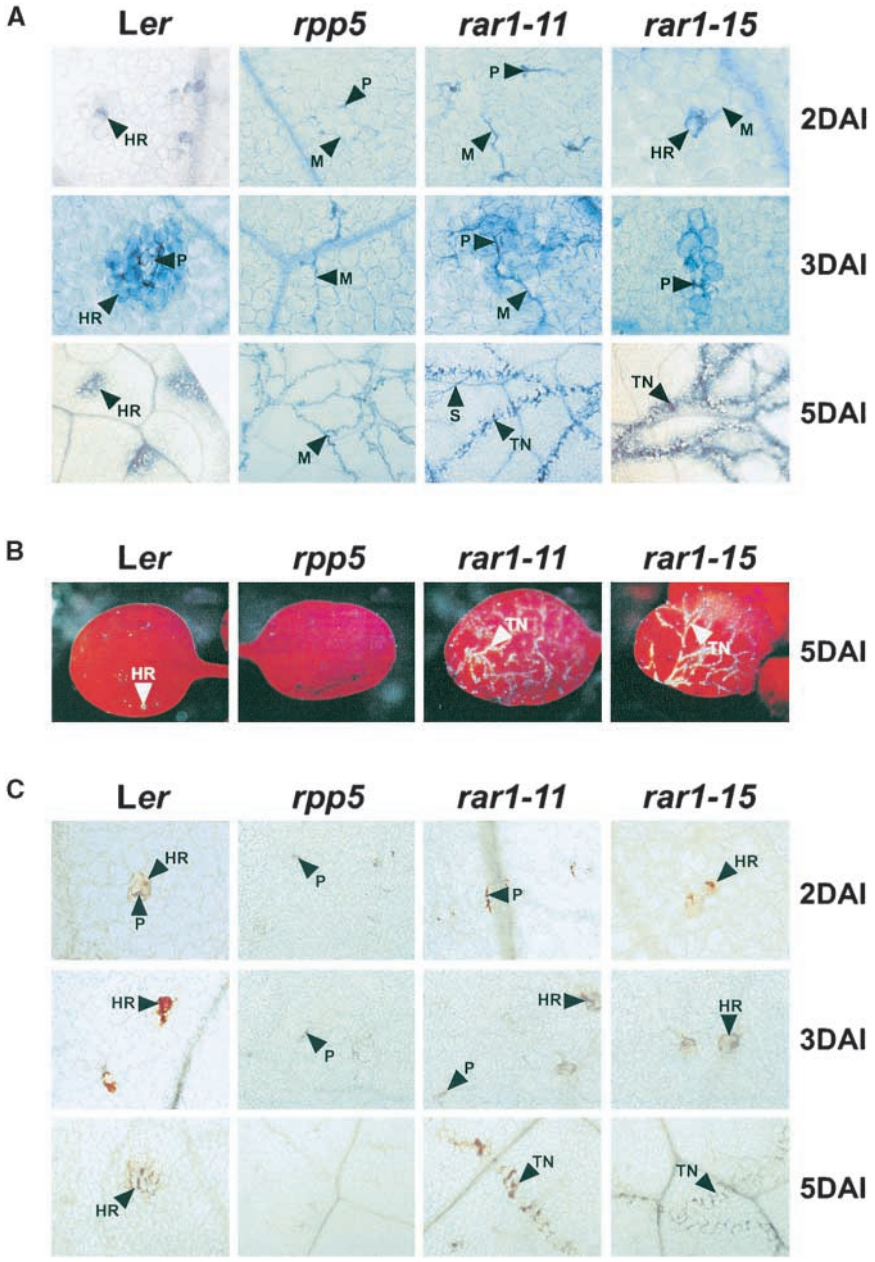

Figure 4. Host Responses and Peronospora Development in *rar1* Mutants.

The images shown are representative of three independent experiments using at least eight leaves per time point for L*er*, *rpp5*, *rar1-11*, and *rar1-15* seedlings after inoculation with Noco2.

(A) Leaves were stained with lactophenol trypan blue at 2, 3, and 5 days after inoculation to reveal necrotic plant cells and pathogen structures.

(B) Cell death–associated autofluorescence viewed under UV light 5 days after Noco2 inoculation.

(C) H_2O_2 accumulation at plant–pathogen interaction sites monitored by 3,3-diaminobenzidine staining of leaves at the same times as in **(A)**.

DAI, days after inoculation; HR, hypersensitive response; M, mycelium; P, penetration site; S, sporangiophore; TN, trailing necrosis. Magnification ×200 (2 and 3 days after inoculation) and ×100 (5 days after inoculation).

Table 2. *R* Gene Requirements for *RAR1*

Pathogen	Isolate/ Strain	*R* gene	NB-LRR Class	*rar1* Phenotype
P. parasitica	Noco2	*RPP5*	TIR	S
	Hiks1	*RPP7*[a]	?	R
	Emco5	*RPP8*	CC	R
	Maks9	*RPP21*	?	(S)
	Cala2	*RPP2*[b]	TIR	R
	Emwa1	*RPP4*[b]	TIR	S
	Cala2	*RPP1A*[c]	TIR	R
P. syringae pv *tomato* (DC3000)	*avrRpm1*	*RPM1*	CC	S
	avrRpt2	*RPS2*	CC	S
	avrRps4	*RPS4*	TIR	S
	avrPphB	*RPS5*[b]	CC	S

All pathogen tests were carried out using *rar1-10*. Plant lines were spray inoculated with *P. parasitica* isolates and resistance or susceptibility scored seven days after inoculation by measuring the extent of host cell death and pathogen sporulation on inoculated leaves. Suspensions of the different *P. syringae* strains were infiltrated into leaves and pathogen growth and disease symptom development recorded over five days. The structures of *RPP8* (McDowell et al., 1998), *RPP2* (Holub, 2001); *RPP1A* (Botella et al., 1998), *RPM1* (Grant et al., 1995), *RPS2* (Bent et al., 1994), *RPS4* (Gassmann et al., 1999) and *RPS5* (Warren et al., 1998) have been described.

[a] *RPP7* in L*er* is defined as a locus cosegregating with Col-0 *RPP7* in >4000 Col-0 × L*er* F2 seedlings but may differ from Col-0 *RRP7* (E. Holub, personal communication).

[b] *R* gene introduced from Col-0 by crossing with L*er rar1-10*.

[c] *R* gene introduced from Ws-0 by crossing. CC, coiled coil; TIR, Toll-Interleukin-1 Receptor; ?, unknown; R, resistant; S, susceptible; (S), partially susceptible.

duced expression of *PR1*, a marker of SA-dependent defenses in plant immunity (Gaffney et al., 1993; Cao et al., 1994; Reuber et al., 1998; Feys et al., 2001), was similar in *rar1* and wild-type responses to virulent DC3000 (Figure 6).

RAR1 Exerts Rate-Limiting Control of *R* Gene–Triggered Defenses

The characterization of null and partially defective Arabidopsis *rar1* mutants revealed a quantitative function of *RAR1* in *R* gene–mediated defenses. Particularly instructive were analyses of *RPP5* resistance in the various *rar1* mutant backgrounds. Here, the extent of plant cell necrosis and pathogen colonization could be quantified readily using a combination of trypan blue staining and visualization of autofluorescence under UV light (Figure 4). We noted that complete loss of *RAR1* function in *rar1-10*, *rar1-11*, and *rar1-13* strongly delayed but did not completely abolish the HR or its accompanying oxidative burst. Similarly, it restricted but failed to halt Peronospora growth. The partial loss of *RAR1* function in *rar1-14* and *rar1-15* resulted in intermediate effects on all of these parameters. The tight correlation between HR intensity, ROI accumulation, and the extent of pathogen containment in *RPP5* resistance suggests that these processes are tightly linked in the defense cascade.

Although we cannot assume that the trailing plant cell necrosis seen at later stages of infection in *rar1* plants (Figure 4) is qualitatively the same as the hypersensitive cell death of wild-type plants, we interpret it as a manifestation of a delayed HR. Consistent with this idea, necrosis occurred only in cells in contact with the invading pathogen. The *RPP5* gene was shown previously to act in a semidominant manner in wild-type plants (Parker et al., 1993). Therefore, both RPP5 and RAR1 are capable of limiting defense signal flux. This raises the question of whether the dependence on *RAR1* could vary with different *RPP5* gene dosages. If *RAR1* is rate limiting in homozygous *RPP5* plants, could it be dispensed with if *RPP5* expression were upregulated? In this regard, it will be useful to test the effect of *rar1* on *RPP5* heterozygotes and on plants overexpressing *RPP5*. We found that the resistance conferred by different *R* genes (*RPM1*, *RPS2*, and *RPS4*) to Pseudomonas also was controlled quantitatively by *RAR1* (Figure 5), although the extent of hypersensitive plant cell death could not be quantified as clearly as in the *RPP5* response.

We conclude that *RAR1* exerts strict control of early signal fluxes leading to the HR triggered by different *R* genes. Whereas the loss of *RAR1* partially suppressed *RPP5* resistance (50% of the loss seen in *rpp5*; Figure 2), it resulted in the complete suppression of *RPM1*, *RPS2*, and *RPS4* resistance to avirulent Pseudomonas. This finding may reflect the different modes of infection by the two pathogens. Alternatively, it might indicate different degrees of *R* gene reliance on *RAR1*, perhaps related to different amounts of RAR1 protein being engaged at a particular regulatory step. Tornero et al. (2002) show that the stability of RPM1 is reduced in a Col-0 null *rar1* mutant. Their finding implies that RAR1 acts at the level of R protein stability before pathogen challenge. This model is not inconsistent with the early, rate-limiting function of RAR1 demonstrated by ourselves and Tornero et al. (2002). Therefore, different degrees of genetic dependence on *RAR1* by *R* genes may reflect the intrinsic efficiency in triggering resistance by a particular R protein. Efficiency could be dictated by R protein abundance in the cell, as implied by the semidominance of *RPP5* (see above), and other factors such as R protein affinity for pathogen avirulence and/or additional signaling components. Resistance mediated by the *RAR1*-independent genes *RPP8* and *RPP1A* resembled that of the wild type in null *rar1* plants in both the timing and extent of the HR and ROI accumulation (data not shown). These R proteins may use different cell death and oxidative burst mechanisms, or they may be sufficiently abundant in the cell to trigger defenses without the involvement of RAR1.

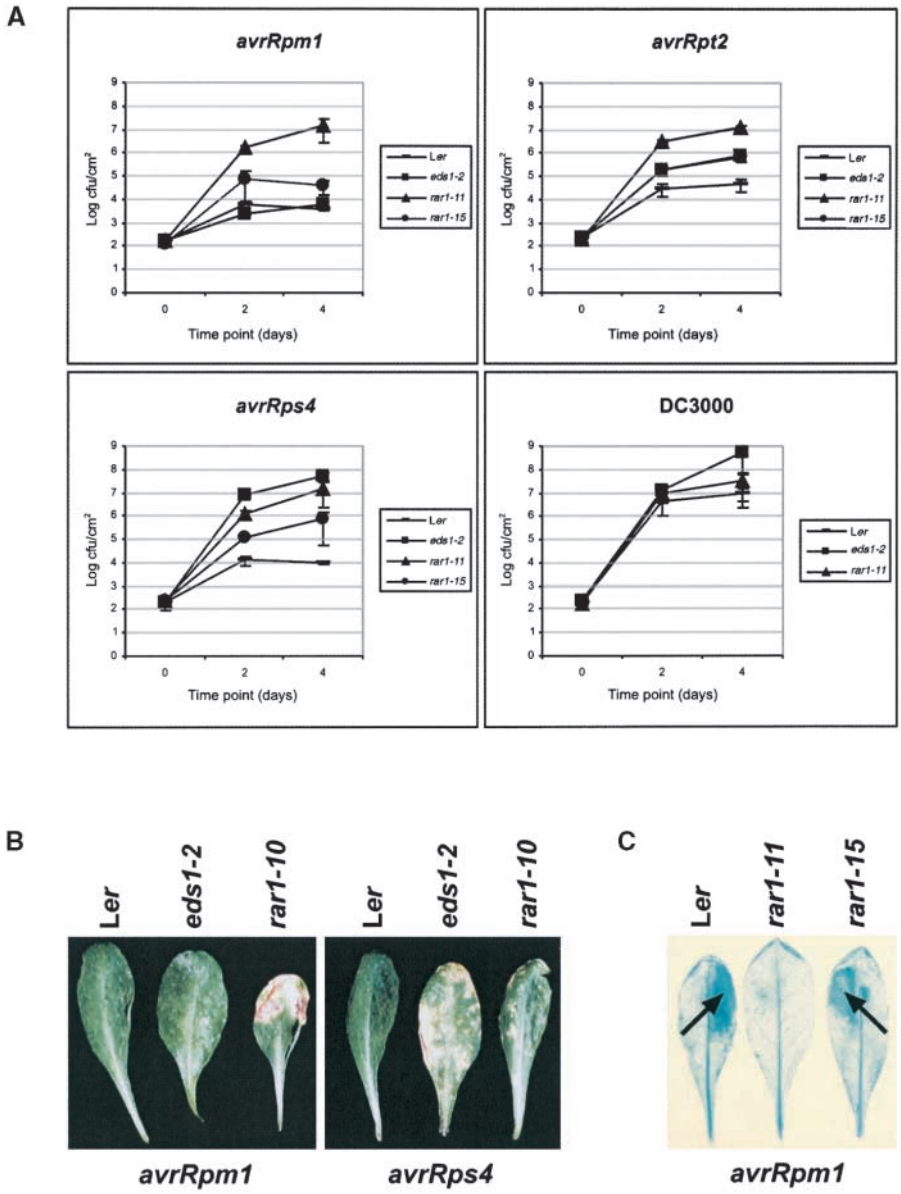

Figure 5. Bacterial Growth and Disease Symptom Formation on *rar1* Leaves.

(A) Growth of Pseudomonas strain DC3000 expressing *avrRpm1*, *avrRps2*, or *avrRps4* or containing an empty vector (DC3000) was measured over 4 days in leaves of L*er*, *eds1-2*, *rar1-11*, and *rar1-15*. Data shown are averages of two independent experiments ±SE. cfu, colony-forming units.

(B) Disease symptoms in L*er*, *eds1-2*, and *rar1-10* caused by Pseudomonas expressing either *avrRpm1* or *avrRps4* at 5 days after leaves were dipped in bacterial suspensions (5×10^7 colony-forming units/mL).

(C) HR development (arrows) in leaves of L*er*, *rar1-11*, and *rar1-15* after hand infiltration of 5×10^6 colony-forming units/mL DC3000/*avrRpm1* and staining at 16 hr with lactophenol trypan blue.

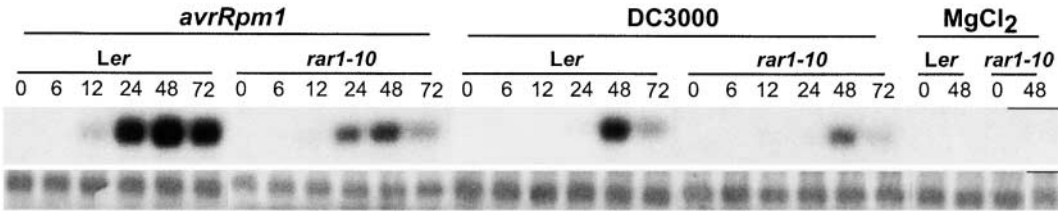

Figure 6. Effect of *rar1* on Pathogen-Induced *PR1* Transcript Accumulation.

Leaves of 5-week-old L*er*, *eds1-2*, and *rar1-10* plants were hand infiltrated with Pseudomonas DC3000 expressing *avrRpm1* or DC3000 containing an empty vector in 10 mM $MgCl_2$ or with 10 mM $MgCl_2$ alone. Total RNA was extracted at 0, 6, 12, 24, 48, and 72 hr after infection, and 20-μg samples were probed on gel blots with a genomic *PR1* DNA fragment. Equal loading was determined by visualization of rRNA on the filter with methylene blue (bottom gel). Three independent experiments gave similar results.

RAR1 Interactions with Other Defense Components

The isolation of definitive null *rar1* mutants (*rar1-11* and *rar1-13*) has helped clarify the issue of whether RAR1 in plants has functions conserved with animal CHORD proteins (Shirasu et al., 1999). The low frequency with which *rar1* mutations were isolated relative to *R* gene mutations in barley (Jørgensen, 1996), coupled with uncertainties about residual barley *rar1-2* activity (Shirasu et al., 1999), left open the possibility that plant RAR1 shares with animal CHORD proteins a vital developmental role, as demonstrated for the *C. elegans* CHORD protein CHP (Shirasu et al., 1999). Because our homozygous null *rar1* mutant lines did not display obvious developmental defects, we favor the idea that plant *RAR1* has evolved a distinct capability in plant defense.

This notion is supported by the recent characterization of barley *rar1-2* as a probable null mutant (Azevedo et al., 2002). Analysis of the partially defective, but full-length, *rar1-14* and *rar1-15* alleles identified in this study permitted further molecular dissection of *RAR1* function. The amino acid exchanges in these mutants were both within the CHORD II domain (Figure 1A, Table 1). In *rar1-15*, the exchange of an invariant Cys for a Tyr in the zinc binding motif led to reduced protein stability (Figure 3), which may contribute substantially to its partial loss of function. In contrast, the rar1-14 protein was as abundant as wild-type RAR1 (Figure 3), indicating that the Glu-to-Lys exchange may perturb a structural or functional role without destabilizing the protein. Regardless of the precise molecular defects of *rar1-14*, its retention of some activity (Figure 2) suggests that the activities of CHORD domains I and II may be at least partially separable. Consistent with this idea, a higher level of sequence conservation between individual CHORD I or II domains of different species than between CHORDs I and II of the same organism suggests a divergence in function (Shirasu et al., 1999). The *rar1-14* mutant line will be a useful tool to test whether CHORD I and CHORD II participate in distinct molecular associations.

Several recent pieces of data provide a link between *RAR1* and a second gene, *SGT1*, in plant resistance. Metazoan CHORD proteins contain a C-terminal extension not found in plant RAR1 sequences that has homology (the CS domain) with a portion of animal SGT1 (Shirasu et al., 1999). In yeast, SGT1 is required for assembly of the kinetochore (CBF3) complex and for SCF (Skp1-Cullin/cdc53-F box) E3 ligase activation through interaction with Skp1 (Kitagawa et al., 1999). In animals cells, SCF E3 ligases recognize specific phosphorylated substrates through their particular F box proteins and target them for ubiquitilation (Deshaies, 1999).

Ubiquitilation of proteins involved in transcription, cell cycle regulation, and other vital cellular functions normally targets them for degradation by the 26S proteasome (Deshaies, 1999; Callis and Vierstra, 2000). In plants, the ubiquitilation of proteins as a means of cellular regulation is poorly understood. However, a molecular connection was established recently between an Arabidopsis SCF complex containing the F box component TIR1 and regulated protein degradation in response to the phytohormone auxin (Gray et al., 2001).

We found that the *rpr1* mutations identified in our *RPP5* suppressor screens (Parker et al., 2000) are defective alleles of *SGT1b*, one of two highly homologous *SGT1* genes in Arabidopsis, suggesting another link between resistance signaling and ubiquitilation of proteins (Austin et al., 2002). *SGT1b* also is required for *RPP7* resistance in Col-0 to Peronospora isolate Hiks1 (Tör et al., 2002). Moreover, transient silencing of *SGT1* in barley single-cell bombardment assays reveals a requirement for *SGT1* in *Mla*-specified powdery mildew resistance (Azevedo et al., 2002).

Although the Arabidopsis *rar1* mutations described here were isolated in the same screens as *sgt1b*, we found only a partial overlap in the spectrum of *R* genes requiring *RAR1* and *SGT1b*, implying that they have both combined and distinct roles in defense (Austin et al., 2002). It is notable that Tornero et al. (2002) present genetic evidence for both cooperative and separate functions of *RAR1* and *NDR1* in different *R* gene–mediated responses. Together, these data

support the existence of a complex matrix of signals that possibly are influenced by the extent of molecular association between various components.

METHODS

Plant Material, Pathogen Strains, and Pathology Tests

The null *rpp5* (Parker et al., 1997) and *eds1-2* (Falk et al., 1999) mutants of *Arabidopsis thaliana* have been described previously. Six *rpr2* (*rar1*) mutants were isolated from fast neutron– or ethyl methanesulfonate–mutagenized Landsberg *erecta* (L*er*) M2 seed obtained from Lehle Seeds (Round Rock, TX). In agreement with the groups of J. Dangl (University of North Carolina, Chapel Hill) and K. Shirasu (Sainsbury Laboratory, Norwich, UK), we have designated L*er rar2-1* to *rpr2-6* as *rar1-10* to *rar1-15*. Wassileskija (Ws-0) *rar1* alleles are designated *rar1-1* to *rar1-9*, and Columbia (Col-0) *rar1* alleles are designated *rar1-20* onward. For pathogenicity tests, plants were grown under a 10-hr photoperiod at 22°C ± 1°C with light intensity of 180 to 250 $\mu E \cdot m^{-2} \cdot sec^{-1}$ and ~65% RH.

Peronospora parasitica and *Pseudomonas syringae* pv *tomato* DC3000 isolates were cultured and prepared for inoculations as described previously (Dangl et al., 1992; Innes et al., 1993). Three-week-old seedlings were sprayed to imminent runoff with 4×10^4 Peronospora conidiospores/mL in sterile distilled water. Conidiospores on leaves of infected plants were quantified 7 days after inoculation by vortexing 20 to 30 seedlings in 2 mL of distilled water. Conidiospores were counted with a hemocytometer using a light microscope at ×200 magnification. Bacterial growth tests were performed by vacuum infiltration of a bacterial suspension (1×10^5 colony-forming units/mL) in 10 mM $MgCl_2$ and 0.01% Silwet L77 (Union Carbide Chemicals and Plastics, Versoix, Switzerland) into leaves of 4- to 5-week-old plants. Disease symptom development was monitored after dipping leaves in a bacterial suspension (5×10^7 colony-forming units/mL) in 10 mM $MgCl_2$ and 0.01% Silwet L77.

Genomic DNA and RNA Preparations

Genomic DNA was prepared from fresh leaf material using the DNeasy 96 Plant Kit (Qiagen, Valencia, CA) in combination with the Retsch MM300 mixer mill (Retsch, Haan, Germany), according to the manufacturer's instructions. Total RNA was extracted from 4-week-old plants using Tri-Reagent (Sigma). Total RNA (20 μg) was loaded per lane on denaturing RNA gels and transferred to nylon membranes. Membranes were stained with 0.03% methylene blue in 0.5 M sodium acetate, pH 5.0, to ensure equal loading. A 1.3-kb *RAR1* genomic DNA fragment (from exons 2 to 6; derived from the cloned *RAR1* gene digested with SspI) was used as a hybridization probe.

RPR2 Mapping Analysis

A backcross mapping population was made by crossing L*er rpr2-1* (*rar1-10*) with Col-0 *glabrous*. Resulting F1 progeny then were crossed to *rpr2-1*. Progeny segregated in a ratio of 1:1 (Noco2-resistant:Noco2-susceptible plants). Susceptible individuals were rescued (sprayed with 0.2 mg/mL Ridomil [Norartis, Greensboro, NC]), and DNA preparations were made of resistant and susceptible plants. Additional populations were generated from resistant individuals that were homozygous for *RPP5* (determined using codominant amplified polymorphic DNA [CAPS] marker g4539 [Parker et al., 1997]) and segregating at *rpr2*. CAPS and simple sequence length polymorphism (SSLP) markers identified in The Arabidopsis Information Resource (TAIR; http://www.aribidopsis.org) were used to position *RPR2* onto the lower arm of chromosome 5. Additional CAPS and SSLP markers were generated from single nucleotide polymorphisms (SNPs) and simple dinucleotide repeats listed on the TAIR World Wide Web site.

In the final stages of mapping, markers were generated from the newly available Cereon Arabidopsis Polymorphism Collection (available through TAIR). *RPR2* was narrowed down to a region of ~220 kb (comprising overlapping transformation-competent artificial chromosome and P1 clones K17N15, K10D11, MIO24, MJM18, and MSG15), based on three remaining recombinant lines. Markers defining this region were as follows: proximal SSLP on K17N15 (41,219 bp), PM57 (5′-TAGGGGAAAATGTAGGATCA-3′) and PM58 (5′-AGCACCTCGTATACACCATC-3′), with polymerase chain reaction (PCR) product sizes of 194 bp (Col-0 *glabrous*) and 181 bp (L*er*); distal SSLP on MSG15 (66,387 bp), PM65 (5′-TCCCTTACTGTCTTGTGGTT-3′) and PM66 (5′-AAAACATGTCATTCGTTTCC-3′), with PCR product sizes of 266 bp (Col-0 *glabrous*) and 243 bp (L*er*). Other markers are available on request.

Amplification and Sequencing of *RAR1*

A 2.2-kb genomic fragment spanning the *RAR1* coding region was amplified from DNA using primers ARAR1 (5′-CCTACCTTCTCAATTCGTCCGATTTCTTC-3′; MIO24, 7095 bp) and ARAR2 (5′-AGAGAGATTCGAGCCGTTCGTTGAGAGTA-3′; MIO24, 4934 bp) prepared from L*er* and the *rpr2* (*rar1*) mutant alleles. PCR errors were minimized by using the Expand High Fidelity PCR System (Roche, Basel, Switzerland). PCR products were sequenced using DNA primer sets spanning the *RAR1* gene. Arabidopsis RAR1 and barley RAR1 protein sequences were aligned with Clustal W (http://www2.ebi.ac.uk/clustalw) and shaded using BoxShade (http://www.ch.embnet.org/software/BOX_form.html).

Selection of *rar1* in Combination with *R* Genes from Other Accessions

Individual lines segregating for Ws-0 *RPP1A* and homozygous for *rar1* were selected from F2 plants derived from a Ws-0 × *rar1-10* cross. A PCR primer pair that detects an SSLP between Ws-0 and L*er* within the Ws-0 *RPP1* locus (5′-GGAATGATGATGTACTGTCCCAACCTCAC-3′ and 5′-ATTCTTGGATCCGCCATATTC-3′) was used in conjunction with *rar1-10* mutant-specific primers. Primers PM81 (5′-CCAGTACAAAAGGCTGTGAT-3′) and PM82 (5′-ACAGTGAAAGAAAAGGGTCA-3′) gave a 195-bp product in the wild type and a 190-bp product in *rar1-10* attributable to a 5-bp deletion (Table 1). Independent lines segregating for *RPP2*, *RPS5*, or *RPP4* in Col-0 and homozygous for *rar1* were selected from Col-0 × *rar1-11* F2 plants using SSLP and CAPS makers within or closely flanking the desired genes. The *RAR1* CAPS marker was derived from a SNP between the L*er* and Col-0 *RAR1* sequence that creates an additional MaeIII site in Col-0 (intron 5). Primers used were PM67 (5′-AAGAACAGATCAAGCAGACC-3′) and PM68 (5′-TCCTTTAGTGCAAGGAGGTA-3′).

Digestion of PCR products with MaeIII gave the following fragment sizes (in base pairs): Col-0, 187, 27, and 138; L*er*, 187 and 165. The RPP2 locus was selected using PCR primer sets AG and g8300 (http://www.aribidopsis.org). PCR primer sets flanking the *RPS5* locus were SSLP marker nga63 and CAPS marker SNP190 (polymorphism detected with RsaI; M.J. Austin, unpublished data). PCR primer sets flanking *RPP4* were AG (http://www.aribidopsis.org) and set 2 (5′-GGGAGATTAAAGAAGCCTTTGC-3′ and 5′-GTGCGG–TTAACTGTTCGGTTACC-3′) detecting an SSLP (Col-0, 1.5 kb; L*er*, 0.9 kb). To test the Col-0 *RPP4* requirement for *RAR1*, it was necessary to select lines that were homozygous Col-0 *rpp8*, because L*er RPP8* also mediates resistance to Peronospora isolate Emwa1 (McDowell et al., 1998). Homozygous Col-0 *rpp8* lines were selected using a *RPP8*-specific CAPS marker (McDowell et al., 1998). Multiple independent lines segregating for the respective *R* genes and homozygous for *rar1* were inoculated with diagnostic Peronospora and Pseudomonas isolates (Table 2).

Protein Immunoblot Analysis

Total protein was extracted from 4-week-old seedlings by grinding with buffer (10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 2.5 mM KCl, 0.14 M NaCl, 5% [v/v] glycerol, 0.2% [v/v] Nonidet P-40 [IGEPAL CA-630, Sigma, Dorset, UK], and protease inhibitor cocktail [Roche]) and centrifugation at 16,000*g* at 4°C. Supernatants were collected, and 30 μg of each sample was resolved on 12% SDS–polyacrylamide gels and blotted onto polyvinylidene difluoride membranes (Amersham). RAR1-specific antibodies raised in rabbits using the full-length recombinant RAR1 protein as antigen have been described elsewhere (Azevedo et al., 2002). Membranes were probed with anti-RAR1 at a dilution of 1:5000, as described by Feys et al. (2001).

Histochemical Analysis of Host–Pathogen Interaction Sites

Plant cell necrosis and Peronospora development were monitored by staining with lactophenol trypan blue as described by Koch and Slusarenko (1990). Staining was performed on leaves of intact plants that had been spray inoculated with Peronospora or on excised leaves on which a 10-μL droplet of conidiospores had been placed. Examination of trypan blue–stained material and detection of H_2O_2 by staining with 3,3-diaminobenzidine was performed as described by Rustérucci et al. (2001).

Accession Numbers

The GenBank accession numbers for the sequences described in this article are AF192262 (Arabidopsis RAR1) and AF192261 (barley RAR1).

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