Organ-Specificity in a Plant Disease Is Determined Independently of *R* Gene Signaling

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Submitted 24 February 2003. Accepted 19 May 2003.

The molecular basis of organ specificity in plant diseases is little characterized. Downy mildew of *Arabidopsis* caused by the oomycete *Hyaloperonospora parasitica* (formerly *Peronospora parasitica*) is characteristically a leaf disease. Resistant host genotypes recognize the pathogen in a gene-for-gene dependent manner and respond with the production of H₂O₂ and the execution of a genetically programmed hypersensitive cell death (HR). We inoculated the roots of *Arabidopsis* genotypes Col-0, Ws-0, and Wei-0 with the NOCO and WELA races of the pathogen and compared the responses with those observed in leaves. Combinations of incompatible genotypes of host and pathogen showed the expected responses of an oxidative burst and the HR in leaves, but surprisingly, roots showed no signs of active defense and appeared completely susceptible to all the *H. parasitica* isolates tested. Reverse transcriptase-polymerase chain reaction showed that the *R* gene *RPP1*, which mediates resistance in leaves of accession Ws-0 to the *H. parasitica* isolate NOCO, was expressed in leaves as well as in roots. Similarly, *NDR1* and *EDS1*, two components of *R* gene-mediated signaling pathways, are also expressed in both tissues. To our knowledge, it has not been previously demonstrated that expression of *R* genes and downstream components of the signaling cascade are not sufficient for the induction of avirulence gene-mediated defense mechanisms in roots.

Additional keywords: hypersensitive response.

In plant-pathogen interactions, specificity exists at various levels. Thus, some pathogens are more or less unspecific regarding their host range, e.g., *Phytophthora omnivora* has more than 2,000 recorded host species. Some pathogens show intermediate specificity and colonize only a few related species, e.g., *Phytophthora infestans* and tomato and potato. At the extreme, in so-called “gene-for-gene” interactions, specificity is restricted to particular genotypes of a single species, as can be shown for *P. infestans* and potato. Thus, the same pathogen (*P. infestans*) shows different levels of specificity (a specificity for the Solanaceae family and at the gene-for-gene level within a species). Yet another level of specificity can be defined within a given compatible gene-for-gene interaction in that the pathogen often shows a preference for colonizing particular plant parts or organs. The “organ specificity” is exemplified in one system of disease classification in which one speaks of e.g., stem cankers, leaf or shoot blights, leaf or fruit spots, root or fruit rots, etc. These organ-specific diseases are caused by

plant pathogens in various taxonomic groups. Although much progress has been made recently in puzzling out the molecular mechanisms governing specificity in gene-for-gene interactions (Glazebrook 2001), at present it is poorly understood why a particular pathogen preferentially infects only some organs of a genetically susceptible host and not the entire plant.

Downy mildew of *Arabidopsis*, caused by *Hyaloperonospora parasitica* (formerly *Peronospora parasitica* [Constantinescu and Fatehi 2002]), is characteristically a leaf-disease, although the stem and petioles can also bear conidiophores and primary infections at the beginning of the annual growing season occur via the roots. Accordingly, germinating oospores that have overwintered in decaying leaves penetrate the root tissue, and the hyphae grow into the stems and leaves where they produce conidiospores and further oospores. In the downy mildew infection cycle, conidiospores infect the leaves of further plants (Koch and Slusarenko 1990; Mauch-Mani and Slusarenko 1993).

The *Arabidopsis*-*Hyaloperonospora* pathosystem is a gene-for-gene interaction in which specific, generally dominant, resistance gene products in the host recognize the products or actions of specific, complementary, avirulence genes in the pathogen (Flor 1947). Inoculation of a large number of different *Arabidopsis* accessions with several isolates of *H. parasitica* has so far revealed more than 20 major resistance gene loci, designated *RPP* genes (recognition of *Peronospora parasitica*), in this host (Reignault et al. 1996; Slusarenko and Schlaich 2003). Several *R* genes, recognizing viral, bacterial, oomycete, and fungal pathogens, have already been cloned (Bent 1996; Glazebrook 2001). Different *R* genes effective against pathogens from various taxonomic groups and from different hosts share structural motifs. Thus, leucine rich repeats (LRR), a nucleotide binding site (NBS), a coiled-coil or a toll-interleukin receptor (TIR) domain are all commonly encountered (Ellis and Jones 1998; Martin 1999). To date, four *R* genes encoding resistance to *Hyaloperonospora parasitica* have been cloned from *Arabidopsis*. All of them contain NBS and LRR domains, but they differ in their NH₂ termini and can be grouped into two classes. *RPP1* and *RPP5* have a TIR domain (Botella et al. 1998; Parker et al. 1997), whereas *RPP8* and *RPP13* have a coiled-coil domain (Bittner-Eddy et al. 2000; McDowell et al. 1998). It has been shown that some *R* genes use different downstream signaling pathways (Warren et al. 1999). So far all tested NBS-LRR containing-*R* genes with a TIR domain have been shown to require *EDS1*, whereas most coiled-coil-containing *R* genes signaled through *NDR1* (Aarts et al. 1998). However, *RPP8* and *RPP13* contain both a coiled-coil domain at their NH₂ terminus and require neither *EDS1* nor *NDR1* (Bittner-Eddy and Beynon 2001; McDowell et al. 2000). *RPP1*, *RPP5*, and *RPP8* are complex resistance gene loci, encoding several functional *R* genes, whereas *RPP13* is a simple locus (Bittner-Eddy et al. 2000;

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Botella et al. 1998; McDowell et al. 1998; Parker et al. 1997). For example, the *RPP13-Rld* allele mediates resistance to the *Hyaloperonospora* isolate WELA in accession Rld-2, while the paralogues *RPP1-WsA*, *RPP1-WsB*, and *RPP1-WsC* mediate

resistance to NOCO in accession Ws-0 (Bittner-Eddy et al. 2000; Botella et al. 1998). Several *R* genes recognize more than one *Hyaloperonospora* isolate. Thus, *RPP1-WsA* recognizes, apart from NOCO2, also EMOY2, CALA2, and MAK59, *RPP1-WsB*

Fig. 1. Interaction phenotypes between compatible and incompatible genotypes in leaves and roots. **A** through **F**, Leaves stained with trypan blue at **A** and **B**, 24, **C** and **D**, 48, and **E** and **F**, 72 h after conidiospores were applied to the upper leaf surface. Panels **A**, **C**, and **E** are from the incompatible interaction between the *Arabidopsis* accession Col-0 and the *Hyaloperonospora parasitica* isolate WELA, whereas panels **B**, **D**, and **F** are from the compatible interaction of accession Wei-0 and the isolate WELA. Similar results were also obtained in other compatible and incompatible interactions. Panels **A**, **C**, and **E** show limited fungal growth and trypan blue staining of hypersensitively responding leaf cells. Panels **B**, **D**, and **F** show successively increasing colonization of leaf tissue without host cell death. **G** through **J**, Roots stained with trypan blue at **G** and **H**, 24 and **I** and **J**, 72 h after conidiospores were applied to the root surface. Panels **G** and **I** are from the incompatible interaction between the *Arabidopsis* accession Col-0 and the *H. parasitica* isolate WELA, whereas panels **H** and **J** are from the compatible interaction of accession Wei-0 and the isolate WELA. Identical results were also obtained in other compatible and incompatible interactions. There is no evidence of hypersensitive cell death. C = conidiospores, Gt = germ tube, Ph = penetration hypha, IH = intercellular hyphae, H = haustorium, and PCD = programmed plant cell death. Scale bars = 20 μ m.

recognizes EMOY2 and MAK5, while *RPPI-WsC* recognizes only NOCO2 (Botella et al. 1998). Resistance against NOCO2, conditioned by any of the three *R* genes of the *RPPI* locus, is dependent on *EDS1* (Aarts et al. 1998; Parker et al. 1996).

In leaves of *Arabidopsis*, the interaction with a genetically incompatible pathogen isolate leads to an oxidative burst and the induction of the genetically programmed cell death response known as the hypersensitive response (HR) (Hammond-Kosack and Jones 1996). In a HR, a small area of host cells in close asso-

ciation with the pathogen undergoes rapid programmed cell death (PCD) usually within 12 to 24 h of inoculation. In the case of obligate biotrophs, like *H. parasitica*, host cell death prevents the establishment of the highly specialized nutritional relationship between host and pathogen and manifests as resistance. In the case of facultative biotrophs, other HR-associated defense responses, such as phytoalexin accumulation, presumably are more important than host cell death per se in limiting pathogen spread (Jabs and Slusarenko 2000). In general, the HR is not

Fig. 2. The oxidative burst is elicited in the incompatible interaction in leaves. Leaves were diaminobenzidine (DAB)-stained **A** and **B**, 8, **C** and **D**, 12, and **E** and **F**, 24 h after conidiospores were applied to the upper leaf surface. Panels **A**, **C**, and **E** are from the incompatible interaction between the *Arabidopsis* accession Col-0 and the *Hyaloperonospora parasitica* isolate WELA. Micrographs depicted in panels **B**, **D**, and **F** are from the compatible interaction of accession Col-0 and the isolate NOCO. Identical results were also obtained in other compatible and incompatible interactions. Note the intense brownish staining of plant cells only in the incompatible interaction, in which plant cells come into intimate contact with infection structures. Roots were DAB-stained **G** and **H**, 4 and **I** and **J**, 12 h after conidiospore were applied to the root surface. Panels **G** and **I** are from the incompatible interaction between the *Arabidopsis* accession Ws-0 and the *H. parasitica* isolate NOCO. Micrographs depicted in panels **H** and **J** are from the compatible interaction of accession Col-0 and the isolate NOCO. Similar results were also obtained in other compatible and incompatible interactions. Note the uniform brownish staining of the central cylinder in all cases. C = conidiospores, Gt = germ tube, Ph = penetration hypha, and H = haustorium. Scale bar = 20 μm.

associated with resistance to necrotrophs (Govrin and Levine 2000; Klement 1986).

In the course of developing a sterile cocultivation procedure to study cellular phenomena in *Hyaloperonospora* infections of *Arabidopsis*, we were surprised to observe an apparent absence of typical resistance phenomena in roots of ostensibly genetically incompatible plants. We investigated this in relation to the possible organ-specific expression of the corresponding *RPP* genes and important signaling components such as *EDS1* and *NDR1*, and our results are reported and discussed here.

RESULTS

Roots offer the advantage of being suitable for the full range of fluorescent dyes available for cell biological studies without the problem of interference from chlorophyll autofluorescence. However, infections of soil-grown plants with *Hyaloperonospora* conidiospores on washed roots were complicated by interference from other microbes. Thus, soil was not a convenient medium for such root studies, so we developed a sterile *Arabidopsis-Hyaloperonospora* pathosystem in petri dishes that was suitable for microscopic studies of the infection process (details below) (Hermanns et al. 2002). Sterile conidia were used to inoculate sterile-grown seedlings that had been placed on microscope slides coated by a thin film of Murashige-Skoog (MS) agar. Inoculated seedlings were placed in an environmentally controlled light cabinet for the infection to develop. All experiments were performed with two different incompatible as well as compatible combinations of *Hyaloperonospora* isolates with *Arabidopsis* genotypes, and representative results are presented. All experiments were carried out at least five times with similar results.

In leaf infections, conidia were placed directly on the leaf surface, whereas in root infections conidia were spread along the roots, often resulting in a small distance from the spore to the root surface that needed to be bridged by the germ tube. Typically, conidia produced germ tubes about six hours after inoculation, and after a short period of growth, these formed

appressoria from which the infection hyphae penetrated into the plant tissue. In a compatible combination, intercellular hyphae and haustoria were formed. In leaves and roots, there was some variation in timing to a given infection stage. Overall, the infection process in roots was similar to that observed in leaves with respect to the type of infection structures formed. Much to our initial surprise, however, in roots, genetically incompatible combinations led to infections indistinguishable in appearance to those seen in genetically compatible combinations.

Programmed cell death.

Leaves. Trypan blue staining was used to stain oomycete structures as well as hypersensitively collapsed plant cells that had undergone PCD. In the incompatible interaction in leaves, epidermal host cell death was observed by 24 h postinoculation (hpi) (Fig. 1A). The empty conidiospore, the germ tube, and the penetration hypha are all clearly visible. The hypersensitive epidermal cell is contrasted to the remaining epidermal cells by a partially blue-stained cell wall, as well as a granular cytoplasm (Fig. 1A). The comparable early stages of the compatible interaction are shown at low magnification in Figure 1B, in which many penetration sites can be seen without any indication of a HR.

By 48 hpi in the genetically incompatible combination, the oomycete had occasionally entered the mesophyll and produced haustoria, while in the compatible interaction hyphal growth and haustoria formation was already quite extensive (Fig. 1C and D).

By 72 hpi, mesophyll cells penetrated by haustoria had undergone PCD (Fig. 1E) whereas, in the genetically compatible combination, the typically branched, "string of sausages" type of intercellular hyphal growth with abundant haustoria and absence of necrotic plant cells was apparent (Fig. 1F).

Root. Interestingly, and in contrast to the situation in leaves, the cytology of infection of roots in genetically incompatible and compatible combinations at 24 and 72 hpi was generally similar (Fig. 1G through J). Germ tubes emanating from conidiospores readily invaded the root tissue and formed intercellular

Fig. 3. Defense signaling gene expression. Reverse transcriptase-polymerase chain reaction (RT-PCR) shows specific expression of *RPP1* in Ws-0 and accession-independent expression of *NDR1* and *EDS1*. RNA was extracted from leaves (lanes 1 to 3) or from roots (lanes 4 to 6) from *Arabidopsis* accessions Wei-0 (lanes 1 and 4), Col-0 (lanes 2 and 5), and Ws-0 (lanes 3 and 6). **A**, Control RT-PCR reactions were carried out with γ -tubulin-specific primers; **B**, RT-PCR reactions were carried out with *RPP1*-WsC-specific primers; **C**, RT-PCR reactions were carried out with *NDR1*-specific primers (genomic DNA was used as template in lane 7); **D**, RT-PCR and nested-PCR reactions were carried out with *EDS1*-specific primers (genomic DNA was used as a template in lane 7).

hyphae from which haustoria that are difficult to discern penetrate the cell wall and invaginate the protoplast of root cells (Fig. 1G and H). Hyphae appeared to grow irrespective of gravitational cues; sometimes basipetally toward the hypocotyl and sometimes acropetally toward the root tip. By 72 hpi, extensive hyphal growth is visible in both genetically compatible and incompatible combinations (Fig. 1I and J). Although there was some background staining of root cells in all panels, there is no evidence of hypersensitive cell death. Similar results were obtained with soil-grown plants (data not shown).

Sometimes, the hyphal cytoplasm appears more dense and the nuclei are clearly stained (Fig. 1I). This occurred independently of compatible or incompatible interactions. Due to experimental limitations, i.e., drying out of the thin agar covering on the microscope slides, observations could not be extended beyond 72 hpi.

Oxidative burst.

Leaves. We tested for another physiological response to infection, occurring in leaves at earlier timepoints than PCD; the generation of reactive oxygen species in an oxidative burst. H_2O_2 production was monitored using diaminobenzidine (DAB) (discussed below), which forms an insoluble brown precipitate in the presence of peroxidases and H_2O_2 . In leaves, striking differences between the incompatible and the compatible interactions were observed. Thus, local H_2O_2 production at the site of attempted penetration in the incompatible interaction was apparent by 8 hpi (Fig. 2A). In those cases in which limited hyphal growth beyond the epidermis occurred, the mesophyll cells also showed an oxidative burst when the pathogen came in contact with them (Fig. 2C and E).

In the compatible interaction, no or only faint DAB staining was observed (Fig. 2B, D, and F). Even cells penetrated by haustoria did not produce H_2O_2 (Fig. 2F). Thus, in leaves, the genetically incompatible interaction is characterized by an oxidative burst that precedes the HR, whereas the compatible host-pathogen combination is characterized by a lack of both reactions.

Roots. In roots, no signs of an oxidative burst were detected at 4, 8, or 12 hpi in either the genetically incompatible (Fig. 2G and h) or in the genetically compatible interaction (Fig. 2I and j). Root cells penetrated by haustoria (Fig. 2J) showed no signs of H_2O_2 production, very much resembling the findings shown for genetically compatible leaves (Fig. 2F). Uniform DAB staining of the central cylinder was observed in all cases, probably due to lignification of the vascular tissue. Also, exogenously supplied H_2O_2 resulted in strong DAB staining of the entire root tissue (data not shown), indicating that peroxidases are ubiquitously expressed in this plant organ.

Expression of defense-related genes.

The absence of an oxidative burst and of PCD in genetically incompatible roots prompted us to investigate whether resistance-related genes, like *R* genes, are expressed in roots. To this end, we carried out reverse transcriptase-polymerase chain reaction (RT-PCR) using total RNA from uninfected *Arabidopsis* accessions Wei-0, Col-0, and Ws-0 with gene-specific primers for *RPP1-WsC*. We used *RPP1* because it is the only published *R* gene relevant for our host-pathogen combinations. As can be seen in Figure 3B, only one PCR product could be obtained when using RNA extracted from either leaves or roots of accession Ws-0 (Fig. 3B, lanes 3 and 6). RT-PCR also yielded bands of the same size when RNA from Wei-0 or Col-0 was used. However, these bands were accompanied by several other larger or smaller bands (Fig. 3B, lanes 1 and 2 and 3 and 4). We sequenced the six bands of the correct size and found frequent mutations in those from accessions Col-0 or Wei-0, indicating that these accessions express nonfunctional versions of *RPP1-WsC*. Conversely, the bands from Ws-0 matched exactly the published sequence.

R gene-dependent signaling is, in most cases studied, mediated via either *NDRI* or *EDS1* gene products. Also *RPP1* signaling is dependent on *EDS1*. Therefore, we tested for *EDS1* and *NDRI* expression via RT-PCR and found these genes to be ubiquitously expressed in leaves and roots of all accessions

Fig. 4. Interaction phenotypes of leaves and roots with a nonhost pathogen. **A**, Leaves of the *Arabidopsis* accession Col-0 infected with *Hyaloperonospora* isolate R1 were diaminobenzidine-stained at 16 h postinoculation (hpi); **B**, leaves of the *Arabidopsis* accession Col-0 infected with *Hyaloperonospora* isolate R1 were trypan blue-stained at 24 hpi. Trypan blue staining at later timepoints gave essentially the same results. **C**, Roots of the *Arabidopsis* accession Wei-0 were infected with *Hyaloperonospora* isolate R1 and trypan blue-stained at 24 hpi; **D**, roots of the *Arabidopsis* accession Wei-0 were infected with *Hyaloperonospora* isolate R1 and trypan blue stained at 72 hpi. C = conidiospores, Gt = germ tube, and Hy = hypha.

(Fig. 3C and D). Again, sequencing of the RT-PCR bands confirmed the identity of the respective genes. As a control for RNA quality, a fragment of γ -tubulin was amplified from all samples (Fig. 3A).

These results suggest that neither lack of *R* gene expression nor the failure to express *NDR1* and *EDS1* as signaling components is responsible for the absence of inducible defenses such as the oxidative burst and the HR in roots.

Nonhost resistance.

On the basis of our results, roots appeared to be susceptible to race-specific pathogens. Therefore, we wanted to extend these findings and investigate the response to nonhost pathogens. To this end we used the *Hyaloperonospora parasitica* isolate R1, which shows race-specific interactions with *Raphanus sativus* but is a nonhost pathogen of *Arabidopsis* (we tested 28 accessions and the *nahG* transgenic line; all were resistant to infection; details are available from the authors by request). We used the same infection methods as described above with R1 on the Col-0, Wei-0, and Ws-0 accessions. In leaves of all three accessions, we found an intense local oxidative burst and HR of a small number of leaf cells around the site of attempted penetration, typical for a nonhost resistance response (Fig. 4A and b). However, even though the growth restriction in leaves was very pronounced, roots could readily be infected. Again, there was no indication of an oxidative burst nor was host cell death but hyphal growth observed. Even though hyphal growth continued over the 3-day observation period (Fig. 4C and D), it appeared to be less vigorous compared with the race-specific isolates WELA or NOCO.

In summary, *Arabidopsis* roots appeared to be susceptible, at least during the 3-day observation period of our sterile-cocultivation system, to infection by compatible and incompatible *Hyaloperonospora* isolates and even nonhost pathogens.

DISCUSSION

General behavior of the pathosystem.

The axenic cocultivation protocol we developed to study the cytology of *Arabidopsis*-*Hyaloperonospora* interactions behaved as expected in leaves. Thus, in leaves, genotype-dependent incompatible or compatible response phenotypes, such as the HR, oxidative burst, or susceptibility (Fig. 1 and Fig. 2, panels A through F) were observed in a manner consistent with earlier reports (Botella et al. 1998; Koch and Slusarenko 1990; Parker et al. 1996). In contrast, inoculation of roots with conidia led to an infection of the host tissue, even when the genotypic combination of host and pathogen ostensibly indicated incompatibility (Figs. 1 and 2 G through J).

Gene expression.

One possible explanation for the lack of induction of *R* gene-dependent resistance responses in roots by genetically incompatible pathogens was that *RPP* genes or genes for essential downstream signaling components might be expressed in an organ- or tissue-specific manner and be absent in roots. This was shown by RT-PCR not to be the case for at least *RPP1*, *NDR1*, and *EDS1* (Fig. 3). Our working hypothesis is that a so-far-unidentified “enabler of defense responses” is principally lacking in roots but present in leaves.

Oxidative burst.

We investigated whether genotypically incompatible *Hyaloperonospora* isolates elicited the oxidative burst in roots and in leaves in our sterile pathosystem. Pharmacological data indicate that blocking the oxidative burst also blocks cell death (Alvarez et al. 1998; Levine et al. 1994) which, however, can

also be uncoupled from resistance (Yu et al. 1998). Leaves, as expected, showed an oxidative burst in incompatible combinations, whereas roots showed no evidence of an oxidative burst (Fig. 2). Similarly, neither wounding nor virulent or avirulent *Pseudomonas syringae* pv. *maculicola* cells caused an oxidative burst in roots, whereas they do in leaves (data not shown).

Evolutionary considerations.

The question arises as to how general a phenomenon the absence of a HR in roots to a genotypically incompatible pathogen might be. The majority of root-infecting fungi are necrotrophs or hemibiotrophs, and resistance to this category of pathogens is generally not associated with a HR (Klement 1986). In whole plant tissues, oxidative burst and the HR responses seem to be largely restricted to leaves or stems (Jabs and Slusarenko 2000). As far as we are aware, there are no reports of an oxidative burst or a HR in roots against plant pathogenic oomycetes, fungi, or bacteria. However, an apparent HR in roots of *Arabidopsis* against incompatible isolates of *Plasmodiophora brassicae* has been reported (Fuchs and Sacristan 1996), as has a HR-like phenomena in the *R* gene-dependent resistance response against root-knot nematodes, such as *Meloidogyne incognita* (Paulson and Webster 1972). However, the large class of cyst nematodes, even where resistance is determined in a gene-for-gene manner as in *Heterodera schachtii* and sugar beet, do not induce a HR in resistant hosts (Cai et al. 1997). Further, even in the case of root-knot nematodes, in which the tomato-*M. incognita* HR (Paulson and Webster 1972) is the best-known example, other cases often seem anomalous. Thus, the HR of tobacco roots to *M. incognita acrita* occurred only after a susceptible response, namely, giant cell formation, had taken place (Powell 1962). It is thus open to question whether reports of cell death in these situations are comparable to the rapid PCD observed in gene-for-gene interactions with microorganisms. In the *P. brassicae*-*Arabidopsis* pathosystem mentioned above, clear monogenically determined resistance against incompatible isolates was demonstrated to be associated with localized lignification and cell death in roots. However, because microscopy was first performed three to four weeks after inoculation, it is unclear whether, since cells often die as a consequence of lignification, the phenomenon was not a HR in the sense of a PCD, but was the result of resistance reactions that, in the long term, lead to the death of the responding cells. This is a subtle but important distinction.

Putting these observations in an evolutionary context, it makes some sense not to have the resistance of roots, which are continually bathed in a hostile soil environment densely packed with microorganisms, based on a PCD response like the HR. Thus, if roots readily executed a HR when confronted with potentially pathogenic microbes, they would be continually dying away and endangering the existence of the plant as a whole. In marked contrast, the microbial density to which leaves are routinely exposed is much lower and small groups of leaf cells can be expended without endangering the survival of the whole organism. Therefore, it may be that the primary resistance determinants in roots are based on other non-HR mechanisms, such as low M_i phytoanticipins (Mansfield 2000), preformed antimicrobial proteins such as defensins, or other non-HR-dependent antimicrobial factors induced after contact with the pathogen. In pea, the root epidermis-specific expression of RH2, which is 95% homologous with the pea pathogenesis-related protein I49a, was suggested as contributing to a constitutive defense barrier in the root epidermis (Mylona et al. 1994). In this vein, it is interesting that high amounts of antimicrobial proteins such as defensins are often present in seeds and roots (Broekaert et al. 2000), both of which usually en-

counter high titers of soil microorganisms. Although constitutively expressed, enhanced synthesis of defensins on infection is dependent upon the jasmonate signaling pathway (Van Loon 2000), and in this regard, it is interesting to note that jasmonate response mutants of *Arabidopsis* (*jar1*) are susceptible to the soil fungus *Pythium irregulare*, whereas wild-type *JAR1* plants are resistant (Staswick et al. 1998).

The special situation of roots in soil in relation to their defense against microorganisms is highlighted in a recent review on the function of root border cells, which are sloughed off the root cap as it grows through the soil (Hawes et al. 2000). Border cells, it seems, act as decoys for fungal attack serving as "cellular goalies" to ward off threats to the apical meristem.

Is there resistance against *H. parasitica* in roots?

Resistance, in the sense of a retardation of pathogen activity, is presumably still happening in the root but might be slow and affect compatible and incompatible isolates in the same way. However, the lack of an oxidative burst and a direct HR, and indeed the apparent susceptibility of *Arabidopsis* roots to ostensibly genotypically incompatible *Hyaloperonospora* isolates, begs the question as to how resistance might be manifested in this situation. Unfortunately, we have never observed any hyphae growing out of the root into the hypocotyl or, further, into the cotyledons and leaves of seedlings. Interestingly, the morphology of the *Hyaloperonospora* hyphae in the roots was often not completely normal. Thus, hyphae appeared more clubbed and irregularly shaped than those in leaves (Fig. 1). It is thus possible that growth of the hyphae in roots is eventually restricted. Unfortunately, at present, we are not able to follow *Hyaloperonospora* isolate growth much beyond 72 h after infection in our sterile pathosystem on the microscope slide used for direct viewing because of the slow dehydration of the thin agar film.

Although *H. parasitica* is essentially a leaf-infecting pathogen, at the beginning of a new growing season, initial infections occur in the soil via roots from germinating oospores that have overwintered in leaf debris from the previous season (Mauch-Mani and Slusarenko 1993). Oospore germination rates are low and variable in the laboratory, and we have not been able to use our sterile pathosystem to study oospore-derived root infections for comparison with infections from conidia.

Synopsis and outlook.

The results reported here touch on the wider problem of how organ-specificity is regulated in plant diseases. This is an area about which very little is known or understood. As our results show, the tissue-specific expression of *R* genes and at least some of the components of defense-associated signal transduction are not sufficient to enable the HR response in all organs. We are currently investigating the possibility that ion fluxes or protein kinases, which act upstream of the oxidative burst, may be compromised in such a way that the roots investigated here cannot mount an effective HR against genotypically incompatible *Hyaloperonospora* isolates. Moreover, we are trying to improve the experimental system so that longer observation peri-

ods become possible. Then, it might be possible to observe the growth of hyphae through the hypocotyl. Particularly in the incompatible interaction, it would be very interesting to observe the point at which active defense responses set in.

MATERIALS AND METHODS

Infections with soil-grown plants.

Arabidopsis seeds were germinated on soil, and seedlings were kept for 10 days under short day conditions (8.5-h light of approximately $90 \mu\text{Em}^{-2}\text{s}^{-1}$). Taking great care not to rupture the young root system, the seedlings were flushed from the soil with running water, were washed extensively with sterile tap water, and were transferred onto microscope slides that were covered with a thin film of half-strength MS agar. Subsequent infection and handling was performed as described for the sterile cocultivation system.

Sterile cocultivation system.

Seeds of *Arabidopsis* accessions Wei-0, Col-0, or Ws-0 were germinated and grown under sterile conditions in vertically oriented petri dishes on a ledge of solidified, half-strength MS medium (Invitrogen, Karlsruhe, Germany) for leaf infections or on tilted glass slides containing a thin film of solidified, half-strength MS medium for root infections. Seedlings had an 8.5-h photoperiod (approximately $90 \mu\text{Em}^{-2}\text{s}^{-1}$) at 22°C and near 100% relative humidity.

H. parasitica was propagated at weekly intervals on sterile, 4-week-old, susceptible host plants. Leaves bearing conidiophores were dipped in sterile tap water, and conidiospores were released by shaking the tube several times. Leaves were infected by placing approximately 20 μl of conidiospore solution on the adaxial leaf surface. Roots were inoculated by pipetting a 5- μl drop of sterile water onto the root surface and spreading this along the entire root system with a conidiophore-bearing leaf (Table 1).

Histochemical procedures.

Staining for fungal growth inside the plants was achieved using the lactophenol trypan blue stain procedure (Keogh et al. 1980). However, the procedure was modified in order to conserve the entire infection structures in place (e.g., spores and germ tube). Thus, the samples were not boiled, but instead, the trypan blue solution was carefully removed after approximately 30 min and was replaced by the chloral hydrate clearing solution.

Production of H_2O_2 was followed with DAB staining (Thordal-Christensen et al. 1997). Briefly, leaves were detached after infection with *Hyaloperonospora* isolates at the indicated time points. They were left overnight in the dark in a 1.6 mg ml^{-1} 3,3'-diaminobenzidine (Sigma, Deisenhofen, Germany) solution. Leaves were destained by boiling for 10 min in 96% ethanol.

Molecular procedures.

For RT-PCR, total RNA was extracted according to standard procedures (Logemann et al. 1987). Briefly, approximately 0.5 g of plant tissue was ground in liquid nitrogen and was added to a tube containing extraction buffer (100 mM LiCl, 100 mM TRIS, pH 8, 10 mM EDTA, 1% sodium dodecyl sulfate), and was phenol/chloroform extracted. After a LiCl precipitation, the RNA pellet was resuspended in water, and sodium acetate was added prior to ethanol precipitation.

RT-PCR reactions were carried out using the Titan one-tube RT-PCR system (Roche, Mannheim, Germany) according to the manufacturer's instructions. From all genes, only a section close to the 3' end was amplified. For *EDS1*, a nested-PCR reaction

Table 1. *Arabidopsis/Hyaloperonospora parasitica* interactions

<i>Arabidopsis</i> accessions	<i>Hyaloperonospora</i> isolates		
	NOCO	WELA	RI
Col-0			
RPP6	compatible	incompatible	nonhost
Wei-0	compatible	compatible	nonhost
Ws-0			
RPP1WsA, WsB, and WsC	incompatible	incompatible	nonhost

following the RT-PCR was necessary to obtain sufficient material for sequencing. The following primer sequences were used. RPP1-WsC forward: GCTTCAGAAGCTATGGGAAGG RPP1-WsC reverse: GGCATTGATAGATGGTGGGAGC NDR1 forward: TTTTCCACCATCAACACGA NDR1 reverse: GCTCCAACCTCAACCCATA EDS1 forward: GGAACCTGGTACAGTCGATGGG EDS1 reverse: GCACCTCGTCAAAAACACCGGC EDS1 nested forward: TCGCTCAATGACCTTGGAGT EDS1 nested reverse: TCAGCTCTCTTGACGTTTGC γ 2-tubulin forward: GCCACACTAGTATCAGGCACC γ 2-tubulin reverse: GAACGCTAACTTAGGATCCGC

The primers were designed, in all cases but the intron-free *NDR1*, to span introns, in order to be able to distinguish by size whether the PCR product was derived from genomic DNA or from mRNA. The identity of the amplified *R* gene, *EDS1*, and *NDR1* bands was confirmed by sequencing.

ACKNOWLEDGMENTS

Research reported here was funded in part by a grant to A. J. Slusarenko from the Deutsche Forschungsgemeinschaft (S130/1-3).

LITERATURE CITED

- Aarts, N., Metz, M., Holub, E., Staskawicz, B. J., Daniels, M. J., and Parker, J. E. 1998. Different requirements for EDS1 and NDR1 by disease resistance genes define at least two *R* gene-mediated signaling pathways in *Arabidopsis*. *Proc. Natl. Acad. Sci. U.S.A.* 95:10306-11.
- Alvarez, M. E., Pennell, R. I., Meijer, P. J., Ishikawa, A., Dixon, R. A., and Lamb, C. 1998. Reactive oxygen intermediates mediate a systemic signal network in the establishment of plant immunity. *Cell* 92:773-784.
- Bent, A. F. 1996. Plant disease resistance genes: Function meets structure. *Plant Cell* 8:1757-1771.
- Bittner-Eddy, P. D., and Beynon, J. L. 2001. The *Arabidopsis* downy mildew resistance gene, *RPP13-Nd*, functions independently of *NDR1* and *EDS1* and does not require the accumulation of salicylic acid. *Mol. Plant-Microbe Interact.* 14:416-421.
- Bittner-Eddy, P. D., Crute, I. R., Holub, E. B., and Beynon, J. L. 2000. RPP13 is a simple locus in *Arabidopsis thaliana* for alleles that specify downy mildew resistance to different avirulence determinants in *Peronospora parasitica*. *Plant J.* 21:177-188.
- Botella, M. A., Parker, J. E., Frost, L. N., Bittner-Eddy, P. D., Beynon, J. L., Daniels, M. J., Holub, E. B., and Jones, J. D. 1998. Three genes of the *Arabidopsis* *RPP1* complex resistance locus recognize distinct *Peronospora parasitica* avirulence determinants. *Plant Cell* 10:1847-1860.
- Broekaert, W. F., Terras, F. R., and Cammue, B. P. 2000. Induced and preformed antimicrobial proteins. Pages 371-478 in: *Mechanisms of Resistance to Plant Diseases*. A. J. Slusarenko, R. S. Fraser, and L. C. Van Loon, eds. Kluwer Academic Publishers, Dordrecht, The Netherlands.
- Cai, D., Kleine, M., Kifle, S., Harloff, H. J., Sandal, N. N., Marcker, K. A., Klein-Lankhorst, R. M., Salentijn, E. M., Lange, W., Stiekema, W. J., Wyss, U., Grundle, F. M., and Jung, C. 1997. Positional cloning of a gene for nematode resistance in sugar beet. *Science* 275:832-834.
- Constantinescu, O., and Fatehi, J. 2002. *Peronospora*-like fungi (Chromista, Peronosporales) parasitic on Brassicaceae and related hosts. *Nova Hedwigia* 74:291-338.
- Ellis, J., and Jones, D. 1998. Structure and function of proteins controlling strain-specific pathogen resistance in plants. *Curr. Opin. Plant Biol.* 1:288-293.
- Flor, H. H. 1947. Host-parasite interactions in flax rust-its genetics and other implications. *Phytopathology* 45:680-85.
- Fuchs, H., and Sacristan, M. D. 1996. Identification of a gene in *Arabidopsis thaliana* controlling resistance to clubroot (*Plasmodiophora brassicae*) and characterization of the resistance response. *Mol. Plant-Microbe Interact.* 9:91-97.
- Glazebrook, J. 2001. Genes controlling expression of defense responses in *Arabidopsis* - 2001 status. *Curr. Opin. Plant Biol.* 4:301-308.
- Govrin, E. M., and Levine, A. 2000. The hypersensitive response facilitates plant infection by the necrotrophic pathogen *Botrytis cinerea*. *Curr. Opin.* 10:751-757.
- Hammond-Kosack, K. E., and Jones, J. D. 1996. Resistance gene-dependent plant defense responses. *Plant Cell* 8:1773-1791.
- Hawes, M. C., Gunawardena, U., Miyasaka, S., and Zhao, X. 2000. The role of root border cells in plant defense. *Trends Plant Sci.* 5:128-133.
- Hermanns, M., Slusarenko, A. J., and Schlaich, N. L. 2002. Lack of active defence responses revealed in a soil-free *Arabidopsis/Peronospora* sterile co-cultivation system. *Plant Prot. Sci.* 38:136-138.
- Jabs, T., and Slusarenko, A. J. 2000. Mechanisms of resistance to plant diseases. Pages 279-323 in: *Mechanisms of Resistance to Plant Diseases*. A. J. Slusarenko, R. S. Fraser, and L. C. Van Loon, eds. Kluwer Academic Press, Dordrecht, The Netherlands.
- Keogh, R. C., Deverall, B. J., and McLeod, S. 1980. Comparison of histological and physiological responses to *Phakopsora pachyrhizi* in resistant and susceptible soybean. *Trans. Br. Mycol. Soc.* 74:329-332.
- Klement, Z. 1986. Hypersensitivity. Pages 149-77 in: *Phytopathogenic Prokaryotes*. M. S. Mount, and G. H. Lacy, eds. Academic Press, New York.
- Koch, E., and Slusarenko, A. 1990. *Arabidopsis* is susceptible to infection by a downy mildew fungus. *Plant Cell* 2:437-445.
- Levine, A., Tenhaken, R., Dixon, R., and Lamb, C. 1994. H₂O₂ from the oxidative burst orchestrates the plant hypersensitive disease resistance response. *Cell* 79:583-593.
- Logemann, J., Schell, J., and Willmitzer, L. 1987. Improved method for the isolation of RNA from plant tissues. *Anal. Biochem.* 163:16-20.
- Mansfield, J. W. 2000. Antimicrobial compounds and resistance. Pages 325-70 in: *Mechanisms of Resistance to Plant Diseases*. A. J. Slusarenko, R. S. Fraser, and L. C. Van Loon, eds. Kluwer Academic Publishers, Dordrecht, The Netherlands.
- Martin, G. B. 1999. Functional analysis of plant disease resistance genes and their downstream effectors. *Curr. Opin. Plant Biol.* 2:273-279.
- Mauch-Mani, B., and Slusarenko, A. J. 1993. *Arabidopsis* as a model host for studying plant-pathogen interactions. *Trends Microbiol.* 1:265-270.
- McDowell, J. M., Cuzick, A., Can, C., Beynon, J., Dangel, J. L., and Holub, E. B. 2000. Downy mildew (*Peronospora parasitica*) resistance genes in *Arabidopsis* vary in functional requirements for NDR1, EDS1, NPR1 and salicylic acid accumulation. *Plant J.* 22:523-529.
- McDowell, J. M., Dhandaydham, M., Long, T. A., Aarts, M. G. M., Goff, S., Holub, E. B., and Dangel, J. L. 1998. Intragenic recombination and diversifying selection contribute to the evolution of downy mildew resistance at the *RPP8* locus of *Arabidopsis*. *Plant Cell* 10:1861-1874.
- Mylona, P., Moerman, M., Yang, W. C., Gloudemans, T., Van de Kerckhove, J., van Kammen, A., Bisseling, T., and Franssen, H. J. 1994. The root epidermis-specific pea gene *RH2* is homologous to a pathogenesis-related gene. *Plant Mol. Biol.* 26:39-50.
- Parker, J. E., Coleman, M. J., Szabo, V., Frost, L. N., Schmidt, R., van der Biezen, E. A., Moores, T., Dean, C., Daniels, M. J., and Jones, J. D. G. 1997. The *Arabidopsis* downy mildew resistance gene *RPP5* shares similarity to the toll and interleukin-1-receptors with *N* and *L6*. *Plant Cell* 9:879-894.
- Parker, J. E., Holub, E. B., Frost, L. N., Falk, A., Gunn, N. D., and Daniels, M. J. 1996. Characterisation of *eds1*, a mutation in *Arabidopsis* suppressing resistance to *Peronospora parasitica* specified by several different *RPP* genes. *Plant Cell* 8:2033-2046.
- Paulson, R. E., and Webster, J. M. 1972. Ultrastructure of the hypersensitive reaction in roots of tomato, *Lycopersicon esculentum* L., to infection by the root-knot nematode, *Meloidogyne incognita*. *Physiol. Plant Pathol.* 2:227-234.
- Powell, N. T. 1962. Histological basis of resistance to root-knot nematodes in flue-cured tobacco. *Phytopathology* 52:25.
- Reignault, P., Frost, L. N., Richardson, H., Daniels, M. J., Jones, J. D., and Parker, J. E. 1996. Four *Arabidopsis* *RPP* loci controlling resistance to the Noco2 isolate of *Peronospora parasitica* map to regions known to contain other *RPP* recognition specificities. *Mol. Plant-Microbe Interact.* 9:464-473.
- Slusarenko, A. J., and Schlaich, N. L. 2003. Downy mildew of *Arabidopsis thaliana* caused by *Hyaloperonospora parasitica* (formerly *Peronospora parasitica*). *Mol. Plant Pathol.* 4:159-173.
- Staswick, P. E., Yuen, G. Y., and Lehman, C. C. 1998. Jasmonate signaling mutants of *Arabidopsis* are susceptible to the soil fungus *Pythium irregulare*. *Plant J.* 15:747-754.
- Thordal-Christensen, H., Zhang, Z., Wei, Y., and Collinge, D. B. 1997. Subcellular localization of H₂O₂ in plants. H₂O₂ accumulation in papillae and hypersensitive response during the barley-powdery mildew interaction. *Plant J.* 11:1187-1194.
- Van Loon, L. 2000. Systemic induced resistance. Pages 521-74 in: *Mechanisms of resistance to plant diseases*. A. J. Slusarenko, R. S. Fraser, and L. C. Van Loon, eds. Kluwer Academic Publishers, Dordrecht, The Netherlands.
- Warren, R. F., Merritt, P. M., Holub, E., and Innes, R. W. 1999. Identification of three putative signal transduction genes involved in *R* gene-specified disease resistance in *Arabidopsis*. *Genetics* 152:401-412.
- Yu, I. C., Parker, J. E., and Bent, A. F. 1998. Gene-for-gene disease resistance without the hypersensitive response in the *Arabidopsis* *dnd1* mutant. *Proc. Natl. Acad. Sci. U.S.A.* 95:7819-7824.