An Investigation into the Involvement of Defense Signaling Pathways in Components of the Nonhost Resistance of *Arabidopsis thaliana* to Rust Fungi Also Reveals a Model System for Studying Rust Fungal Compatibility

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Seventeen accessions of *Arabidopsis thaliana* inoculated with the cowpea rust fungus *Uromyces vignae* exhibited a variety of expressions of nonhost resistance, although infection hypha growth typically ceased before the formation of the first haustorium, except in Ws-0. Compared with wild-type plants, there was no increased fungal growth in *ndr1* or *eds1* mutants defective in two of the signal cascades regulated by the major class of *Arabidopsis* host resistance genes. However, in the Col-0 background, infection hyphae of *U. vignae* and two other rust fungi were longer in *sid2* mutants defective in an enzyme that synthesizes salicylic acid (SA), in *npr1* mutants deficient in a regulator of the expression of SA-dependent pathogenesis related (*PR*) genes, and in NahG plants containing a bacterial salicylate hydroxylase. Infection hyphae of *U. vignae* and *U. appendiculatus* but not of *Puccinia helianthi* were also longer in *jar1* mutants, which are defective in the jasmonic acid defense signaling pathway. Nevertheless, haustorium formation increased only for the *Uromyces* spp. and only in *sid2* mutants or NahG plants. Rather than the hypersensitive cell death that usually accompanies haustorium formation in nonhost plants, *Arabidopsis* typically encased haustoria in calloselike material. Growing fungal colonies of both *Uromyces* spp., indicative of a successful biotrophic relationship between plant and fungus, formed in NahG plants, but only *U. vignae* formed growing colonies in the *sid2* mutants and cycloheximide-treated wild-type plants. Growing colonies did not develop in NahG tobacco or tomato plants. These data suggest that nonhost resistance of *Arabidopsis* to rust fungi primarily involves the restriction of infection hypha growth as a result of defense gene expression. However, there is a subsequent involvement of SA but not SA-dependent *PR* genes in preventing the *Uromyces* spp. from forming the first haustorium and establishing a sufficient biotrophic relationship to support further fungal growth. The *U. vignae*-*Arabidopsis* combination could allow the application of the powerful genetic capabilities of this model plant to the study of compatibility as well as nonhost resistance to rust fungi.

form of plant resistance to disease but is still poorly understood in comparison with the resistance shown by specific genotypes within an otherwise susceptible host species. This latter resistance is commonly controlled by parasite-specific resistance (*R*) genes, but the role of these genes in nonhost resistance is controversial and probably varies with the plant-pathogen combination (Heath 2001). Although constitutive plant features may contribute to nonhost resistance, they rarely can completely account for the lack of successful pathogen infection (Heath 1997). Inducible defense responses also are involved, and in the absence of any gene-for-gene recognition system, these defenses are likely to be elicited more nonspecifically in a manner analogous to the recognition of pathogen molecules in the innate immune response of animals (Aderem and Ulevitch 2000).

Biotrophic pathogens, with their sophisticated type of pathogenesis that keeps plant cells alive and minimizes tissue damage in susceptible hosts, are good pathogens for the study of nonhost resistance. Nonhost resistance to biotrophic fungal pathogens that penetrate epidermal cells directly, such as the powdery mildew fungi and the basidiospore-derived parasitic stage of rust fungi, is typically expressed as penetration failure (Mellersh et al. 2002). Potentially more experimentally informative, however, is the second parasitic stage of the rust fungi derived from urediospore infection. This parasitic stage is not only the most economically important with respect to rust diseases of crop plants, but it also involves a larger defined set of developmental stages during the fungal infection process (Fig. 1). After spore germination on a leaf, topographical features of the plant surface may be used by the fungus to locate and recognize stomata prior to going through a defined series of morphological stages required to enter the stomatal opening and form the first haustorium within a living host mesophyll cell (Heath 1997). This haustorium is assumed to take up sufficient nutrients from the invaded plant cell to allow further intercellular fungal growth and haustoria formation. Previous studies comparing host and nonhost resistance to rust fungi have shown that nonhost resistance is typically expressed before the formation of the first haustorium (Heath 1981). This resistance sometimes involves prepenetration events, such as the poor location and recognition of nonhost stomata, and invariably involves restricted growth if the fungus enters the substomatal cavity. In such cases, fungal growth may be inhibited before the formation of a haustorial mother cell (Fig. 1A), or the haustorial mother cell may fail to penetrate a plant cell (Fig. 1B). In contrast, *R* gene-controlled host resistance is almost invariably expressed after the formation of the first haustorium, often in

Nonhost resistance, exhibited by entire plant species to a particular microbial pathogen, is the most common and durable

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the form of the hypersensitive death of the invaded cell, and prehaustorial signs of resistance are rare. This morphological distinction between host and nonhost resistance has been used to detect host cultivars that have unusually high levels of “background,” nonhostlike, rust resistance that may be more durable than resistance controlled by *R* genes (Anker and Niks 2001).

Although nonhost resistance expressed after the rust fungus has entered the plant’s intercellular spaces involves active plant responses, some of which modify the surrounding plant cell walls (Fink et al. 1991; Perumalla and Heath 1991), the signaling systems involved in their induction are unknown. This study exploits available *Arabidopsis thaliana* mutants and rust fungi to demonstrate differences in the nonhost signaling pathways from those involved in host resistance to *Arabidopsis* pathogens and to reveal an *Arabidopsis*–rust fungus system that could facilitate the study of compatibility to these economically important biotrophic pathogens, even though *Arabidopsis* currently has no available rust disease.

RESULTS

Nonhost resistance of dicotyledonous plants to rust fungi has been most extensively studied using *Uromyces vignae*, the cowpea rust fungus (Heath 1997), so this fungus was initially used to investigate the expression of nonhost rust resistance in *Arabidopsis*.

Different accessions of *Arabidopsis* exhibit different microscopical expressions of nonhost resistance.

Seventeen accessions of *Arabidopsis* were inoculated with *U. vignae*, and infection sites were examined microscopically nine days after inoculation, by which time a large colony (several mm in diameter) with extensive intercellular hyphae and intracellular haustoria should have developed in susceptible host cowpea plants (Fig. 1F). Table 1 shows that all tested *Arabidopsis* accessions exhibited the typical expression of nonhost resistance to rust fungi: namely, the cessation of fungal growth at most infection sites during growth of the infection hypha and before the formation of the first haustorium. However, there were differences between accessions. In some,

penetration into the leaf was rare, suggesting that the fungus may have responded incorrectly to topographical cues on the leaf surface, as has been demonstrated for this fungus on other nonhost plants (Heath 1974). Although infection hyphae of the fungus were short (less than about 40 μ m long) and brown in most accessions (Fig. 2A), in some, the fungus was colorless and much longer (60 μ m or more). Localized modifications of the mesophyll walls closest to the fungus involving browning or papilla formation were common in some accessions but rare in others. Only in one accession, Ws-0, were any haustoria seen, and these were always encased in calloselike material, as is commonly seen in situations in which the haustorium is killed (Škalamera and Heath 1996). The contents of the invaded cells were usually not brown or granular, indicating that they had not undergone a hypersensitive cell death. The fungus at some haustorium-containing infection sites continued to grow to produce a small colony with up to six haustoria. Most of these haustoria also became encased, and the invaded cells did not die, but some colonies were associated with one or two dead cells typical of the hypersensitive response. The presence and frequency of fungal colonies varied dramatically between experiments and between plants within an experiment, indicating the strong influence of local physiological factors in influencing the outcome of the Ws-0–fungus interaction. Nevertheless, these observations demonstrate that, although *Arabidopsis* is a nonhost species for *U. vignae*, in genotypes or situations in which prehaustorial defenses are less effective, the fungus is able to develop a sufficiently compatible biotrophic relationship with the plant cell to nutritionally support a small amount of fungal growth.

Several *Arabidopsis* mutations affect prehaustorial fungal growth but few allow haustorium formation and posthaustorial development.

Arabidopsis plants (Col-0 or Ws-0) with mutations in signaling pathways known to be involved in host disease resistance were inoculated with *U. vignae*, and the length of the infection hypha, including the substomatal vesicle and any haustorial mother cell (Fig. 1B), at each of 100 infection sites was measured. Compared with Col-0, in which the fungus was often restricted to a small brown substomatal vesicle without much elon-

Fig. 1. Diagrammatic representation of a leaf cross-section illustrating the different stages of *Uromyces vignae* development in *Arabidopsis* wild-type, mutant, or NahG plants or after cycloheximide treatment (CH) of wild-type Col-0 plants. **A**, and **B**, Typical fungal growth for the plants or treatments listed. **C**, Plants or treatments in which over 30% of infection hyphae formed at least one haustorium. **D**, **E**, and **F**, Plants or treatments in which stages of fungal development were attained by over 5% of haustorium-containing infection sites. Ssv = substomatal vesicle; ih = infection hypha; hmc = haustorial mother cell; and * = in some experiments only.

gation into an infection hypha (Fig. 2A), there was a significant increase in infection hypha length in all Col-0-derived mutants, except the *ndr1* mutant, which is defective in a gene required for resistance controlled by most known examples of a subclass of *R* genes with nucleotide-binding site-leucine-rich repeat (NBS-LRR) motifs (Glazebrook 2001). Compared with its parent Ws-0, the *eds1* mutant, which is defective in a gene required for resistance controlled by the other subclass of NBS-LRR *R* genes, also did not support increased infection hypha growth.

In the first experiment, reported in Table 2, for plants with the Ws-0 background, about 20% of fungal infection hyphae formed one or two encased haustoria in the *eds1* mutant and in its comparable wild-type plant. Small fungal colonies were seen only in one leaf in the mutant (Fig. 1D), most likely the result of the variability in this feature typical of this accession. In contrast, haustoria were rare in wild-type Col-0 and, despite the increase in infection hypha length in several mutants, haustoria were only observed in significantly higher numbers in *sid2* plants (Table 2). Also, only in this mutant were a significant number of fungal colonies observed, some of which were very large (Fig. 1F) and were equivalent to those seen in the susceptible cowpea host. As *SID2* encodes the enzyme isochorismate synthase involved in salicylic acid (SA) synthesis (Wildermuth et al 2001), we examined fungal growth in transgenic NahG plants containing a bacterial enzyme, salicylate hydroxylase, that degrades SA. In the

Col-0 background, infection hypha growth was almost equivalent to that seen in susceptible cowpea leaves, and over 70% of infection hyphae formed the first haustorium and continued growing to produce colonies (Table 2), of which almost half were very large (Fig. 2B) and resembled those in susceptible host leaves, except for the presence of encased haustoria. Infection hypha length was also increased in NahG plants in the Ws-0 background, as was the incidence of growing colonies, although, in the experiment reported in Table 2, haustorium and colony frequency was high even in the wild-type control. Interestingly, colonies in Ws-0 NahG plants were never as large as those seen in Col-0 NahG leaves and were commonly associated with more signs of a hypersensitive response (one or more dead plant cells) (Fig. 2C as compared with Fig. 2B). Figure 1 shows a diagrammatic summary of *U. vignae* growth in the various *Arabidopsis* plants tested.

Different species of rust fungi respond differently to *Arabidopsis* signaling mutants.

To see if the growth of other species of rust fungi are affected by the *Arabidopsis* mutations that affect *U. vignae*, *sid2*, *npr1*, and *jar1* mutants and NahG plants were inoculated with the bean rust fungus *U. appendiculatus* and the sunflower rust fungus *Puccinia helianthi*. Increased infection hypha growth of *U. appendiculatus* was seen in NahG plants and all the mu-

Table 1. Interactions of *Uromyces vignae* with *Arabidopsis* accessions.

Accession	Stock Number ^a	Origin if known	Interaction ^b
Col-0	...	...	Short, brown IH, some wall responses
Ler-0	...	...	Short, brown IH
Ws-0	...	U.S.S.R	Medium-long IH, some sites with one or two encased haustoria or small colonies.
Condara	CS6175	Tadjikistan	Short, brown IH, wall responses common
Hodja-Obi-Garm	CS6178	Tadjikistan	Short, brown IH
Rld-2	CS6927	Russia	Low penetration, medium length IH
Sn	CS6181	Czechoslovakia	Long IH
Tsu-1	CS6926	Japan	Short-long IH, shorter ones brown
Oy-1	CS6929	Norway	Short-long IH
Est	CS6173	Germany	Short brown IH
Ak-1	CS6602	Germany	Short, shriveled, brown IH, wall responses common
Wei-0	CS6182	Switzerland	Short-long, shriveled IH
Bs-1	CS6627	Switzerland	Short IH, some brown, some wall responses
Ag-0	CS6601	France	Low penetration, short IH
Bl-1	CS6615	Italy	Short-long IH
Can-0	CS6660	Canary Islands	Short IH, few brown
Cvi-0	CS6675	Cape Verde Islands	Low penetration, short brown IH, wall responses common

^a From *Arabidopsis* Biological Resource Center, Ohio State University.

^b IH = intercellular infection hypha. Wall responses include browning and localized thickening of the mesophyll walls adjacent to the infection hypha. Haustoria were seen only in Ws-0.

Fig. 2. Light micrographs of infection sites of *Uromyces vignae* in whole, cleared leaves. **A**, A small brown fungal substomatal vesicle (f) in a Col-0 plant with modifications (arrows) of the surrounding plant cell walls. **B**, A small part of a large fungal colony formed in Col-0 NahG plants. Nearly every plant cell contains a fungal haustorium, some of which are encased (solid arrows) completely or partially (*). A few small haustoria are not encased (dotted arrows). **C**, One of the larger fungal colonies seen in Ws-0 NahG plants, with a few encased haustoria (one shown at h) and a small amount of intercellular hyphae (arrow) as well as an associated brown plant cell (B).

tants, as had been seen for *U. vignae*, and haustorium formation similarly was dramatically increased in *sid2* and NahG plants (Table 3). However, unlike *U. vignae*, *U. appendiculatus* did not form any colonies in the *sid2* mutant, although it did form small ones in NahG plants. *P. helianthi* differed from both of the *Uromyces* species in that no increased infection hyphae length was seen in *jar1* plants (Table 3). Infection hyphae of *P. helianthi* only occasionally formed a haustorium in *npr1* plants, but no haustoria were seen in the other mutants or in NahG plants, even when the fungus had grown long enough to form a haustorial mother cell.

The effect of the NahG transgene on *U. vignae* is restricted to *Arabidopsis*.

To see if *U. vignae* forms large growing colonies in other plant species containing the NahG transgene, tobacco and tomato plants containing this transgene were inoculated with this fungus. As shown in Table 4, about half of the infection hyphae formed a single haustorium in both wild-type and NahG tomato plants, and there was no difference between plants in the average length of infection hyphae. An occasional small colony was seen only in wild-type plants. In wild-type tobacco, fewer infection hyphae produced a single haustorium than was seen in wild-type tomato and even less formed haustoria in NahG plants. No fungal colonies were seen in any tobacco plant. Haustorium-containing cells in both plant species were brown, indicative of a hypersensitive response.

Cycloheximide treatment mimics the effect of the NahG transgene.

Various treatments that inhibit prehaustorial defense responses in plants allow *U. vignae* to produce growing colonies in nonhost plants (Heath 1979). Therefore, the effect of a pre-inoculation treatment with cycloheximide was examined with all three rust fungi on *Arabidopsis* Col-0 (Table 5). The inhibitor significantly increased infection hypha length only for *U. appendiculatus* and *P. helianthi*. Cycloheximide treatment increased the number of infection hyphae that produce a haustorium only for the two *Uromyces* species and allowed small or medium-sized fungal colonies to be formed only by *U. vignae* (Fig. 1).

Table 2. Growth of *Uromyces vignae* in *Arabidopsis* mutants and transgenic NahG plants

Plant genotype	Length (μm) ^a	% Infection sites	
		With at least one haustorium	With a fungal colony ^b
Col-0	24.4	1	0
Col-0 NahG	65.0*	76*	72*
<i>sid2</i>	53.3*	32*	7*
<i>npr1</i>	31.8*	2	0
<i>jar1</i>	47.9*	9	0
Col-0 ^c	28.8	0	0
<i>npr1</i>	40.5*	0	0
<i>ndr1</i>	33.2	0	0
Ws-0 ^d	39.4	18	0
<i>eds1</i>	34.2	24	0.8
Ws-0	39.9	43	24
Ws-0 NahG	46.8*	57	72*

^a Mean infection hypha length, including the substomatal vesicle and any haustorial mother cell. * = significantly different from the comparable wild type (Col-0 or Ws-0) values ($P \leq 0.05$).

^b With at least three haustoria and associated intercellular mycelium.

^c The plants in the rest of this column were grown under 16-h days rather than 12-h days.

^d Ws-0 is the wild type for *eds1*; values for the Ws-0 mutant or transgenic plants were not compared to Col-0.

DISCUSSION

Whether nonhost resistance to fungal pathogens involves the same type of gene-for-gene recognition that is involved in many examples of host genotype resistance is a matter of debate and probably depends on the specific plant-parasite combination (Heath 2001). However, there is evidence that certain genes may have a role in both *R* gene-mediated resistance and selected examples of nonhost resistance in the same plant, suggesting some commonalities may exist in the resistance process (Peart et al. 2002). Flax is the only dicot from which rust resistance genes (against the flax rust fungus) have been cloned and these belong to the NBS-LRR class of *R* genes (Anderson et al. 1997). Two subclasses of these genes, containing a coiled-coil or a TIR motif, are found in *Arabidopsis*, and the signal transduction cascades that they govern often require the expression of *NDR1* or *EDS1* genes, respectively (Glazebrook 2001). In the current study of the nonhost resistance of *Arabidopsis* to the cowpea rust fungus *U. vignae*, mutations in these *NDR1* or *EDS1* genes had no significant effect on fungal growth. However, as there is at least one signal transduction cascade in *Arabidopsis* that transduces NBS-LRR *R* gene signals without involving *EDS1* or *NDR1* (Glazebrook 2001), it cannot be stated for certain that there is no involvement of *R* gene signaling pathways in nonhost resistance to *U. vignae*.

The uredial stage of most rust fungi is established via stomatal penetration, and studies suggest that compatibility with the host species requires the ability to i) avoid or negate prehaustorial defenses within the substomatal cavity of the plant, ii) breach the mesophyll plant cell wall to form the first haustorium, and iii) establish a biotrophic relationship with the living plant cell to sustain further fungal growth (Heath 1981). Nonhost resistance typically is expressed prehaustorially (Heath 1981), and the current study indicated that this was also the case in a variety of *Arabidopsis* accessions inoculated with *U. vignae* or Col-0 inoculated with two other species of rust fungi. However, there were microscopical indications that the plant-fungus interactions were not identical in each accession, and the variable degrees of prehaustorial resistance seen in Ws-0, between plants and between experiments, suggest that defensive features in this plant are unusually sensitive to physiological factors.

That prehaustorial resistance is the result of active plant defenses is demonstrated by the fact that it is usually compromised by transient heat or inhibitor treatments that reduce plant defense responses (Heath 1979). Two signal transduction pathways, one

Table 3. Growth of *Uromyces appendiculatus* and *Puccinia helianthi* in *Arabidopsis* mutants and transgenic NahG plants

Plant genotype and fungal sp. ^a	Length (μm) ^b	% Infection sites	
		With at least one haustorium	With a fungal colony ^c
<i>U. appendiculatus</i>			
Col-0	40.5	3	0
NahG	73.3*	71*	15*
<i>sid 2</i>	62.3*	19*	0
<i>npr1</i>	53.6*	5	0
<i>jar 1</i>	46.8*	4	0
<i>P. helianthi</i>			
Col-0	38.5	0	0
NahG	49.5*	0	0
<i>sid 2</i>	44.4*	0	0
<i>npr1</i>	48.4*	3	0
<i>jar 1</i>	37.6	0	0

^a All plants were grown under 12-h days.

^b Mean infection hypha length, including the substomatal vesicle and any haustorial mother cell. * = Significantly different from the comparable Col-0 data ($P \leq 0.05$).

^c With at least three haustoria and associated intercellular mycelium.

SA-dependent and one dependent on either jasmonic acid (JA), ethylene, or both, have been implicated in the induction of defenses involved in *Arabidopsis* resistance to pathogens other than rust fungi (Thomma et al. 2001), and the current study of mutants in the Col-0 background strongly suggests the involvement of an SA-dependent pathway in nonhost resistance to all three rust fungi tested. Increased prehaustorial growth was seen in *sid2* mutants with a disruption in an enzyme involved in SA synthesis (Wildermuth et al. 2001) and in mutant *npr1* plants. NPR1 functions downstream of SA accumulation in resistance controlled by the TIR-class of *R* genes (Rairdan and Delaney 2002) and is required for SA-induced expression of pathogenesis-related (PR) proteins (Glazebrook 2001). Therefore, the data suggest a role for SA-inducible PR proteins in nonhost resistance of *Arabidopsis* to rust fungi, and in support of this conclusion, there is evidence that *Uromyces* species are sensitive to apoplastic PR proteins, including PR-1, a marker protein for the SA signaling pathway (Rauscher et al. 1999). However, increased infection hypha growth in the *jar1* mutant suggests that the JA-dependent pathway also has a role in resistance to the two *Uromyces* species studied, although not to *Puccinia helianthi*. The JA-dependent pathway is typically involved in responses to wounding and insect damage (Thomma et al. 2001), and it may be that differences in the trauma caused by infection hyphae of different rust fungi can result in the induction of non-SA-dependent, as well as SA-dependent, signal transduction cascades.

Intracellular haustorium formation is a process that requires signal exchange between plant and fungus, as well as the negation of defenses triggered by the attempted penetration of the plant cell wall (Heath 1997). For all three rust fungal species in the present study, an increase in infection hypha length did not necessarily lead to the formation of a haustorium, suggesting that the prehaustorial defenses that inhibit infection hypha growth are not necessarily the same as those that prevent haustorium formation. In particular, the lack of any effect of the *npr1* mutation on haustorial frequency suggests that absence of the PR proteins elicited by the NPR1 branch of the SA-dependent signaling pathway does not promote haustorium formation. Nevertheless, haustorium formation by *Uromyces* spp. was apparently promoted by the reduced levels of SA found in the *sid2* mutant and in NahG Col-0 plants containing a bacterial SA-degrading enzyme. The fact that a single prepenetration treatment of wild-type plants with cycloheximide also increased haustorium formation by the two *Uromyces* spp. indicates that the factor or factors that normally prevent haustorium formation by these fungi require protein synthesis.

The general inability of *P. helianthi* to form haustoria in any plant in this study reflects earlier experiments that demonstrated

the inability of this fungus to form haustoria in nonhost plants treated transiently with treatments to reduce prehaustorial defenses (Heath 1979), and ultrastructural studies have suggested that haustorium formation fails because of the strong expression of wall-associated defense responses during attempted cell wall penetration (Wood and Heath 1986). Such responses are not seen when the *Uromyces* species occasionally form haustoria in untreated nonhost plants (M. C. Heath, unpublished data). These observations indicate that there may be significant differences between rust fungal genera in their ability to suppress penetration-associated defenses in nonhost plants.

In *sid2*, NahG, and cycloheximide-treated wild-type Col-0 *Arabidopsis* leaves, some infection hyphae of *U. vignae* not only formed a haustorium but the fungus continued to grow to form colonies that were particularly large in NahG plants. A similarly dramatic increase in susceptibility of NahG *Arabidopsis* plants to a bacterial *Arabidopsis* nonpathogen, *Pseudomonas syringae* pv. *phaseolicola*, has recently been suggested, in part because of the failure of resistance to be similarly compromised in SA-reduced *sid2* plants, to be due to the increased presence of SA degradation products such as catechol (van Wees and Glazebrook, in press). This hypothesis may not be applicable in the current system, as growing colonies of *U. vignae* were seen in *sid2* plants, albeit not as large as those seen in NahG plants. Nevertheless, preliminary studies in our lab have shown that treatment with the shikimic acid pathway inhibitor glyphosate reduces the frequency of growing colonies in *sid2* plants and increases the frequency of a hypersensitive response to the first-formed haustorium. Such results support the possibility that phenolic compounds in addition to SA are involved in the susceptibility of *Arabidopsis* to *U. vignae*.

The formation of growing fungal colonies of *U. vignae* in *sid2*, NahG, and cycloheximide-treated *Arabidopsis* plants indicates that haustoria were able to establish a growth-supporting biotrophic relationship with the invaded plant cells, and as seen in Col-0 NahG plants, such growth could be as great as that seen in susceptible cowpea leaves, even though the fungus did not sporulate. This ability to grow well in *Arabidopsis* seemed to be restricted to *U. vignae*, as *U. appendiculatus* only formed small growing colonies in NahG plants and *P. helianthi* never formed a colony in any plant. Significantly, *P. helianthi* has never been induced to form growing colonies in nonhost species, whereas *U. vignae* can be induced to do so in several nonhosts (Heath 1979, 1980). Haustoria of rust and powdery mildew fungi are known to induce a state of susceptibility (known as "accessibility") in invaded and surrounding host plant cells (Heath and Škalamera 1997). Conceivably, the development of this state requires a minimum period of haustorium-plant cell interaction that is normally curtailed in nonhost plants by rapid defense responses to the first-formed haustorium. However, any delay in these responses as the result of cycloheximide treatment or the absence of SA could allow this accessible state to be achieved, and further fungal growth to occur, but only if relevant fungal and nonhost plant attributes were sufficiently compatible. In this scenario, it appears that *U. vignae* has attributes that allow it to be biotrophically compatible with a wider variety of nonhost plants than *U. appendiculatus* or *P. helianthi*. This conclusion is reminiscent of those from recent studies that demonstrate increased bacterial growth in some, but not all, bacteria-nonhost combinations when defense responses are suppressed (Peart et al. 2002).

The most common response of nonhost plants if a rust fungus manages to form its first haustorium is the hypersensitive death of the invaded plant cell (Heath 1977), as was seen in the current study in the interaction between *U. vignae* and tomato or tobacco. Therefore, the general lack of a hypersensitive response (HR) in haustorium-containing *Arabidopsis* cells of any

Table 4. Growth of *Uromyces vignae* in NahG tomato and tobacco plants

Plant ^a	Length (μm) ^b	% Infection sites	
		With at least one haustorium	With a fungal colony ^c
Tomato			
Wild type	45.0	68	1
NahG (8721) ^d	50.3	49	0
NahG(8723) ^d	52.9	52	0
Tobacco			
Wild type	59.3	19	0
NahG	66.4	5 ^e	0

^a All plants were grown under 12-h days.

^b Mean infection hypha length including the substomatal vesicle and any haustorial mother cell.

^c With at least three haustoria and associated intercellular mycelium.

^d From seed from two different NahG parents.

^e Significantly different from the comparable wildtype data ($P \leq 0.05$).

genotype is remarkable. Instead of a HR, the haustorium became encased in calloselike material. Such encasement of a penetrating object is the default response of plant cells and suppressing this response is seemingly part of the haustorial activities required to develop a successful biotrophic relationship with the plant cell (Heath and Škalamera 1997). The large size of some of the haustoria in wild-type Ws-0 argues against the encasement forming rapidly enough to shield the plant cell from HR-eliciting fungal activities and, instead, suggests that *Arabidopsis* may not be as sensitive to these activities as other plant species. Interestingly, the expression of a putative callose synthase gene in an *Arabidopsis* mutant with increased callose levels is reduced by the presence of the NahG salicylate hydroxylase (Østergaard et al. 2002). Therefore, the large *U. vignae* colonies seen in NahG plants in the current study may, in part, reflect a slower rate of haustorial encasement.

The current study indicates that normal levels of SA in Col-0 *Arabidopsis* are necessary both for the expression of prehaustorial defenses to this fungus and for the successful expression of posthaustorial defenses if a haustorium forms. Interestingly, the rather striking effect of the NahG transgene in the *U. vignae*-Col-0 *Arabidopsis* nonhost system was not seen in *U. vignae*-infected NahG tomato or tobacco, suggesting that factors other than SA are more important in nonhost resistance to this fungus in some plant species. There also appears to be some variability between *Arabidopsis* accessions, as *U. vignae* colonies in Ws-0 NahG plants were not as large and were associated with more signs of plant resistance than when the salicylate hydroxylase was present in the Col-0 background.

Overall, our results suggest that there is no single mechanism of nonhost resistance of *Arabidopsis* to rust fungi, and SA may play a greater or lesser role depending on the fungal species. Although *Arabidopsis* rust resistance may differ from that of other nonhost plants, the *U. vignae*-*Arabidopsis* nonhost pathosystem is valuable as a comparison to the many studies of host resistance in this plant. However, perhaps more significantly, despite the importance of rust diseases in agriculture and the desirability of exploiting the useful characteristics of *Arabidopsis* to study plant-rust fungal interactions, there is no available fungus that causes a natural *Arabidopsis* rust disease. Therefore, the ability of *U. vignae* to form colonies in NahG plants similar to those seen in the susceptible host suggest that the *Arabidopsis*-*U. vignae* combination could be a model system in which to apply the powerful genetic capabilities of *Arabidopsis* to the study of not only nonhost resistance but also compatibility to rust fungi.

MATERIALS AND METHODS

Plant material.

Arabidopsis thaliana seedlings were grown on Murashige-Skoog media (Sigma-Aldrich, Ontario, Canada) supplemented with 0.8% phytagar (Gibco, Rockville, Maryland, U.S.A.) in a growth chamber maintained at 20 to 22°C and illuminated at 150 $\mu\text{E m}^{-2}\text{s}^{-1}$ for ten days prior to being transferred to sterile soil. Plants were then generally grown under 12-h days, but some mutants, particularly *npr1*, were stunted under this light regime, so they and their wild-type controls were grown under 16-h days. Tomato and tobacco plants were planted directly in sterile soil and were kept in a growth chamber at 20 to 22°C and illuminated for 16 h per day at 150 $\mu\text{E m}^{-2}\text{s}^{-1}$.

The accessions of *A. thaliana* listed in Table 1 were obtained from the Arabidopsis Biological Resource Center (Ohio State University). Col-0 (Columbia-0) and Ler-0 (Landsberg erecta line) were donated by R. Cameron, University of Toronto. The following transgenic and mutant plants were used: seed from two transgenic NahG plants containing a bacterial salicylate

hydroxylase from a line derived from wild-type Moneymaker tomato, courtesy of J. D. G. Jones (The Sainsbury Laboratory, Norwich, U.K.); transgenic NahG-10 Xanthi tobacco and NahG *Arabidopsis* Col-0 line B15 and Ws-0 line 3A from Syngenta Agribusiness Biotechnology Research Inc. (Research Triangle Park, NC, U.S.A.), and mutants *sid2-1* (SA induction deficient 2) from C. Nawrath (University of Fribourg, Switzerland), *npr1* (nonexpressor of *PR 1*) from X. Dong (Duke University, Durham, NC, U.S.A.), *jar1* (jasmonic acid resistant 1) from P. Staswick (University of Nebraska, Lincoln, NE, U.S.A.), *ndrl-1* (nonrace-specific disease resistance 1-1) from B. J. Staskawicz (University of California, Berkeley, CA, U.S.A.), and *eds1-1* (enhanced disease susceptibility 1-1) and Ws-0 from J. Parker (Plant Bioscience Ltd., Norwich, U.K.).

Fungal inoculations.

Urediospores of the cowpea rust fungus (*Uromyces vignae*, race 1), bean rust fungus (*Uromyces appendiculatus*, race 0), or the sunflower rust fungus (*Puccinia helianthi*, race 2) were washed with 0.01% Tween for 30 s (2 min for *U. appendiculatus*) to remove self inhibitors of germination and then were rinsed several times with sterile distilled water. Urediospores were applied to the upper surface of leaf seven or eight (leaf 1 being the youngest) of 25-day-old *Arabidopsis* plants and the first emerged true leaves of 20-day-old tomato and tobacco plants using a soft artist's paintbrush. Following inoculation, plants were misted with distilled water and were put into a dark humidity chamber overnight prior to being returned to the lighted growth chamber.

Pharmacological treatments.

In some experiments, prior to inoculation, the intercellular spaces of leaves were injected with cycloheximide (1 $\mu\text{g ml}^{-1}$ in distilled water) (Sigma-Aldrich) or sterile distilled water (as a control), using a 30-gauge needle and a 1 cc syringe. Leaves were inoculated once they no longer had a water-soaked appearance (usually about 1 h after injection).

Leaf fixation and examination.

Leaf pieces were generally harvested nine days postinoculation to allow the maximum extent of fungal growth to have taken place. Leaves were fixed and decolorized in boiling 95% (vol/vol) ethanol before being cleared in saturated chloral hydrate and being mounted on glass slides in modified Hoyer's medium (Cunningham 1972). Leaves were examined using a Reichert-Jung Polyvar microscope (Reichert AG, Vienna, Austria) equipped with differential interference contrast optics.

Table 5. Effect of cycloheximide (1 $\mu\text{g ml}^{-1}$) on the growth of three rust fungi in wild-type Col-0 *Arabidopsis*

Fungus and treatment ^a	Length (μm) ^b	% Infection sites	
		With at least one haustorium	With a fungal colony ^c
<i>Uromyces vignae</i>			
Water Control	42.8	17	0
Inhibitor	40.0*	48*	19.5*
<i>U. appendiculatus</i>			
Water Control	71.7	19	0
Inhibitor	77.1*	50*	0
<i>Puccinia helianthi</i>			
Water Control	38.7	9	0
Inhibitor	55.5*	4	0

^a All plants were grown under 12-h days.

^b Mean infection hypha length including the substomatal vesicle and any haustorial mother cell. * = significantly different from the comparable water control data ($P \leq 0.05$).

^c With at least three haustoria and associated intercellular mycelium.

Image acquisition.

Images were acquired using a Nikon (Nikon Instruments Inc., Melville, NY, U.S.A.) color digital still camera, model DXM1200, and the ACT-1 image acquisition software.

Data collection and analysis.

Data are presented as means of fungal lengths from 100 fungal penetration sites (25 sites collected on each of four leaves per treatment) or means of percentages from each leaf with a given phenotype for each treatment. Length data were compared using a Mann-Whitney rank sum test. When necessary, percentage data were normalized prior to being compared using a *t*-test. All experiments were performed at least twice with similar results; typical data from one experiment are shown.

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