Characterization of Nonhost Resistance of *Arabidopsis* to the Asian Soybean Rust

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Asian soybean rust (ASR), caused by *Phakopsora pachyrhizi*, is a devastating disease of soybean. We report the use of the nonhost plant *Arabidopsis thaliana* to identify the genetic basis of resistance to *P. pachyrhizi*. Upon attack by *P. pachyrhizi*, epidermal cells of wild-type *Arabidopsis* accumulated H₂O₂, which likely orchestrates the frequently observed epidermal cell death. However, even when epidermal cell death occurred, fungal hyphae grew on and infection was terminated at the mesophyll boundary. These events were associated with expression of *PDF1.2*, suggesting that *P. pachyrhizi*, an ostensible biotroph, mimics aspects of a necrotroph. Extensive colonization of the mesophyll occurred in *Arabidopsis pen* mutants with defective penetration resistance. Although haustoria were found occasionally in mesophyll cells, the successful establishment of biotrophy failed, as evidenced by the cessation of fungal growth. Double mutants affected in either jasmonic acid or salicylic acid signaling in the *pen3-1* background revealed the involvement of both pathways in nonhost resistance (NHR) of *Arabidopsis* to *P. pachyrhizi*. Interestingly, expression of *AtNHL10*, a gene that is expressed in tissue undergoing the hypersensitive response, was only triggered in infected *pen3-1* mutants. Thus, a suppression of *P. pachyrhizi*-derived effectors by *PEN3* can be inferred. Our results demonstrate that *Arabidopsis* can be used to study mechanisms of NHR to ASR.

Additional keywords: *eds1-2*, *jar1-1*, *sid2-1*.

Increasing human mobility facilitates spread of phytopathogenic organisms into new habitats. In their new environments, pests and pathogens face plant communities that are not adapted to them. Introduced pathogens may thus cause disastrous epidemics in new habitats. One recent example for such a scenario is the spread of aggressive Asian soybean rust (ASR) isolates to the New World. Soybean rust is caused by the basidiomycete fungus *Phakopsora pachyrhizi* and has been known for decades in the Eastern Hemisphere. In 1914, the pathogen was first reported on the legume *Pachyrhizus erosus* by H. and P. Sydow (Koch et al. 1983). The pest started a world-wide journey from Taiwan and spread to Australia (presence reported in 1934), Africa (approved in 1978), and South America (reported in 1980) (Koch et al. 1983). In November 2004, *P. pachyrhizi* landed in the continental United States

(Stokstad 2004). ASR was found in 2005 on kudzu (*Pueraria lobata*) (Harmon et al. 2006), an immigrant weed that serves as an alternate host that enables the pathogen to overwinter in the soybean-free time of the year (Christiano and Scherm 2007). Since ASR aggressively defoliates fields of soybean, it has received top ranking for soybean diseases in the Americas. However, regional spread of ASR has, so far, been limited in North America, presumably due to low inoculum available in 2005 and dry weather conditions in 2006 (Christiano and Scherm 2007).

The genus *Phakopsora* contains about 80 species. Six species have been described that parasitize legumes, including *P. pachyrhizi* (Asian-Australian species) and *P. meibomiae* (tropical-Latin American species), which each have an exceptionally wide host range, including soybean (Ono et al. 1992). Natural infections with *P. pachyrhizi*, for example, have been reported in 31 species from 17 genera of legumes, and artificial infections have been successful on 60 plant species in 26 additional genera (Rytter et al. 1984). In addition to its wide host range, ASR's modus of direct penetration of the epidermis is a feature that is uncommon for rust uredospores, which mostly enter the leaf by producing appressoria over stomata. However, direct penetration is a feature of monokaryotic basidiospores, e.g., of the cowpea rust fungus *Uromyces vignae* (Heath 1997; Mellersh and Heath 2001).

Uredospores of *P. pachyrhizi* germinate and form appressoria even on artificial substrates such as dialysis membranes, confirming that plant-derived signals are not needed for these developmental steps. However, as the frequency of appressorium formation corresponded to the pore size of membranes, the involvement of thigmodifferentiation rather than chemodifferentiation has been suggested (Koch and Hoppe 1988). During penetration of soybean epidermal cells, a funnel-shaped structure was formed in the appressorium, providing a cell-wall continuum with the penetration hyphae (Koch et al. 1983). The latter transversed the epidermis and entered the spongy mesophyll, leaving behind a disorganized, subsequently collapsing, epidermal cell. Penetration hyphae differentiated to form primary and secondary hyphae. Finally, haustorial mother cells were formed that introduced thin infection pegs into mesophyll cells, followed by haustorial initials and mature haustoria (Koch et al. 1983). The development of uredosori and uredospores completes the pathogenesis of *P. pachyrhizi* on susceptible soybean leaves.

ASR is currently managed by fungicides. However, development of fungicide-resistant ASR strains must be expected, and the economic costs of repeated fungicide treatments are not negligible. In addition, traditional breeding strategies so far only provided soybean varieties that withstood particular races of the fungus, and after their introduction to the field, these traits were soon overcome (Bonde et al. 2006; Hartman et al.

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2005; Twizeyimana et al. 2007). In order to identify novel potential resistance traits to ASR, we elucidated a nonhost resistance (NHR) response to the fungus. NHR confers durable protection to all genotypes of a given plant species against all races of a given pathogen species in natural as well as agricultural systems. Therefore, NHR could be considered a useful resource to improve crop resistance (Ellis 2006; Heath 2000; Mysore and Ryu 2004; Thordal-Christensen 2003).

Over the past decade, *Arabidopsis thaliana* has emerged as a valuable model to study NHR in plants (Holub and Cooper 2004; Yun et al. 2003). Recently, a series of penetration mutants (*pen1*, *pen2*, and *pen3*) were identified in the model plant *Arabidopsis thaliana*, allowing considerable progress towards understanding NHR. Each mutant enabled entry of nonhost powdery mildew pathogens into epidermal cells at a higher frequency. *PEN1* encodes a syntaxin that might play a role in vesicle trafficking to the plasma membrane (Assaad et al. 2004; Collins et al. 2003). *PEN2* is a glycosyl hydrolase, while *PEN3* represents an ABC transporter that seems to act cooperatively with *PEN2*. It has been proposed that *PEN2* turns on an inactive precursor of an unknown metabolite that prevents fungal penetration after its release to the apoplast by action of *PEN3* (Lipka et al. 2005; Stein et al. 2006). Finally, concomitant knock-out of pre- and postpenetration defense led to full compatibility between *Arabidopsis* and nonhost powdery mildew pathogens (Lipka et al. 2005).

Recently, the interaction of *Arabidopsis* with the stomata-penetrating cowpea rust fungus (*Uromyces vignae*) was developed as a model to study NHR against rusts (Mellersh and Heath 2003). Here, we present the novel interaction between *Arabidopsis* and the direct-penetrating rust fungus *P. pachyrhizi*, which will allow further insight into NHR of *Arabidopsis* against this aggressive, introduced pathogen. Additionally, the use of a model plant has helped to overcome the limitations of the soybean-ASR pathosystem. Thus, light microscopical and histochemical analyses were used to follow *P. pachyrhizi* infection in various *Arabidopsis pen* single and double mutants. In this way, we were able to successively eliminate particular components of the nonhost defense machinery, allowing us to move increasingly towards a compatible *P. pachyrhizi*-*Arabidopsis* interaction. Finally, we elucidated successful traits of the NHR repertoire of *Arabidopsis* that could potentially be genetically engineered into soybean crops to combat ASR.

Fig. 1. The *pen3-1* mutant of *Arabidopsis* shows macroscopic symptoms after infection with *Phakopsora pachyrhizi*. Representative leaves of wild-type (Col-0) plants (upper panel) and *pen3-1* mutants (lower panel) were taken 10 days after inoculation with uredospores (right) or mock treatment (left).

RESULTS

Nonhost resistance to ASR in *Arabidopsis*.

In this work, we established a tractable ASR-*Arabidopsis* interaction in the lab that will allowed us to identify important components of the NHR of *Arabidopsis* to *P. pachyrhizi*. As expected from a bona fide nonhost plant, the pathogen did not cause macroscopic lesions or sporulate on any of the 28 *Arabidopsis* accessions tested in this study (a complete list can be found below). This finding confirmed that *Arabidopsis* can be regarded as a nonhost for ASR.

Next, we tested various *Arabidopsis* mutants with known defects in pathogen resistance. Of these, only *pen3-1* displayed macroscopic disease symptoms in response to ASR infection (Fig. 1). Infected *pen3-1* leaves initially became chlorotic and then turned necrotic (Fig. 1). Similar disease symptoms have been reported for *pen3-1* after infection with other pathogens, including *Phytophthora infestans* and *Botrytis cinerea* (Stein et al. 2006).

Cytology of the ASR-*Arabidopsis* interaction.

To investigate the interaction between ASR and *Arabidopsis* at the cellular level, leaves of *Arabidopsis* plants infected with *P. pachyrhizi* were stained with trypan blue before microscopy. After germination, the fungus penetrated epidermal leaf cells directly (Fig. 2A and B). This behavior contrasts markedly with that of the majority of rust fungi, which enter plants via stomata (Koch et al. 1983). On wild-type plants, *P. pachyrhizi* invaded epidermal cells by forming an appressorium followed by a penetration hypha that grows into the lumen of the epidermal cell (Fig. 2B). Death of the penetrated cell was characterized by granulation of the cytoplasm and the accumulation of trypan-blue stain. (Keogh et al. 1980). In all the 28 different wild-type *Arabidopsis* accessions tested in this study, the fungus exited the epidermal cell but did not grow further, either inter- or intracellularly, into the mesophyll. In contrast to the wild type, *pen3-1* epidermal cells did not always stain with trypan blue after penetration by *P. pachyrhizi* (Fig. 2C through F). In addition, independent of whether the penetrated epidermal cell stained with trypan blue, fungal growth proceeded intercellularly into the mesophyll tissue (Fig. 2E and F). There, cells around the hyphae displayed pronounced papilla formation (Fig. 2E), which also holds true for infection sites on the *pen2-1/pad4-1* mutant (Fig. 2G). Fungal hyphae continued their growth in the intercellular spaces (Fig. 2F) and, in a few cases, formed haustorium-like structures in mesophyll cells (Fig. 2H). Together, the observed infection process and the infection structures discovered in *Arabidopsis* wild-type or *pen3-1* mutant plants seemed to be similar to those known from the interaction of *P. pachyrhizi* with its soybean host (Koch et al. 1983). However, in all *Arabidopsis* genotypes tested, the fungus never sporulated, indicating that the NHR of *Arabidopsis* to *P. pachyrhizi* is determined after penetration, probably by inhibition of extensive fungal spread either into or within the mesophyll or both.

Histochemical classification of interaction sites.

To evaluate the development of *P. pachyrhizi* on various *Arabidopsis* mutants, interaction phenotypes were quantitatively assessed at the cellular level (Fig. 3). Leaves of infected *Arabidopsis* plants were harvested at the fourth day postinoculation (dpi) and were stained with trypan blue. For quantitative evaluation, we discriminated seven classes of interaction sites that encompassed pre- and postpenetration events. The first two classes included germinated spores without (class 1) or with (class 2) appressoria at the end of germ tubes but with no visible plant-defense reaction. Class 3 was defined by the pres-

ence of papillae beneath the site of attempted penetration. Class 4 comprised epidermal cells that had undergone cell death, as determined by trypan-blue staining. In classes 5 and 6, epidermal cells were trypan-blue stained and the fungus developed hyphae into the intercellular spaces of the mesophyll without (class 5) or with (class 6) trypan blue-stained mesophyll cells. Finally, interaction sites were observed at which mesophyll cells were stained with trypan blue but the penetrated epidermal cell was not (class 7).

Quantitative assessment of single-cell interaction sites.

Based on the above classifications, a clear picture emerged for fungal development on each *Arabidopsis* genotype (Fig. 3). Essentially, we did not observe significant differences between different accessions or mutants, either in the germination rate of uredospores or the formation of appressoria. Papillae formation as a defense response was mainly observed on plants without a *pen* mutation, e.g., *Arabidopsis* Col-0 and Col-3 *gll*. However, in about 60% of the interaction events between these two genotypes and *P. pachyrhizi*, fungal penetration into epidermal leaf cells caused a cell-death response (Fig. 3). No substantial fungal growth was observed in mesophyll tissue of Col-0 and Col-3 *gll* plants. This observation indicates the presence of an effective NHR mechanism in wild-type *Arabidopsis*. In contrast, fungal growth was observed in 30 and 40% of the interaction sites in the mesophyll of *pen1-1* and *pen2-1* mutants, respectively, and this was mostly associated with cell death in the epidermis. Basically, the same holds true for the *pen1-1/pen2-1* double mutant (Fig. 3). Infections of *pen3-1* by *P. pachyrhizi* resulted in a strong reduction in the number of infection sites at which fungal development was stopped shortly after the epidermis had been breached. Rather, in the *pen3-1* mutant, *P. pachyrhizi* extensively colonized the mesophyll tissue. The latter was true for nearly 60% of the interaction sites, with 18% of the mesophyll cells undergoing cell death in response to fungal colonization (Fig. 3). This category of interaction sites was barely detected on *pen1-1* and *pen2-1* mutant plants. Essentially, based on the microscopic analysis, the colonization of the *pen2-3/pen3-2* double mutant resembled that of *pen3-1*. The same was true for the *pen2-1/pad4-1* double mutant (Fig. 3).

Next, we investigated the progression of ASR development on *Arabidopsis* by recording the cellular interaction phenotype in leaves harvested at 1, 4, and 7 dpi. This revealed differences between genotypes as early as 1 dpi (data not shown). While fungal hyphae were not found in the mesophyll of wild-type *Arabidopsis* plants up to 4 dpi, they were observed at 10% of the interaction sites by 7 dpi. However, at these sites, fungal growth was limited. In contrast, pronounced fungal mycelium was found as early as 1 dpi in the mesophyll of mutant *pen3-1* and *pen2-1/pad4-1*. Notably, trypan-blue staining in the mesophyll was not observed until 4 dpi (data not shown).

Single cell-interaction phenotypes between *pen3-1* double mutants and ASR.

The above results support a crucial role for PEN3 in controlling the NHR of *Arabidopsis* to *P. pachyrhiza*. However, even on *pen3-1* mutants, fungal progression stopped after colonization of confined patches within the mesophyll. As a next step, we tested whether combined mutations in *PEN3*, and genes involved in well-described defense signaling pathways would compromise this mesophyll resistance. Specifically, we investigated the *jar1-1* (jasmonic acid [JA] response) (Staswick et al. 1998, 2002) mutant of *Arabidopsis*, which is compromised in JA signaling. In addition, two mutants with defects in the salicylic acid (SA) signal transduction pathway have been included in the assay, i.e., *sid2-1* (salicylic-acid deficient)

(Wildermuth et al. 2001) and *eds1-2* (enhanced disease susceptibility) (Falk et al. 1999; Parker et al. 1996). Leaves of the single-mutant plants evaluated in this experiment did not differ from the wild type after inoculation with *P. pachyrhizi* (data not shown).

For microscopic analysis, inoculated leaves were harvested at 2 dpi and were subjected to trypan-blue staining. Cellular interaction phenotypes were categorized as described above. For simplicity, class 1, which did not vary in proportion among the different host genotypes under investigation, is not shown in Figure 4. Furthermore, interaction sites with trypan blue-stained epidermal cells and hyphal growth in the mesophyll (formerly classes 5 and 6) were combined in a single class. A high frequency (approximately 50%) of interaction sites were observed on Col-0 plants at 2 dpi, in which the fungus had built an appressorium without causing any detectable staining

Fig. 2. Impaired basal defense in *Arabidopsis* mutants allows *Phakopsora pachyrhizi* to enter the mesophyll. **A** and **B**, Infection sites were photographed at 1 d postinoculation (dpi) of Col-0 wild-type plants and **C** through **F**, *pen3-1* mutants with uredospores. **G** and **H**, Infection on mutant *pen2-1/pad4-1* at 4 dpi. Leaves were stained with trypan blue before analysis with differential interference contrast (**A** through **F**) or bright-field (**G**, **H**) microscopy. Series of consecutive optical cuttings were made across particular infection sites starting with the focus on uredospores (**A**, **C**), then moving stepwise through the epidermis (**B**, **D**), and finally to the mesophyll (**E**, **F**, **G**, **H**). Structures reminiscent of a haustorium were found on mutant *pen2-1/pad4-1* (**H**). sp = uredospore, app = appressorium, ph = cross-section of penetration hypha in epidermal cells, pap = papilla, prh = primary hypha in the intercellular space of the mesophyll, and hau = haustorium-like structure. Nomenclature of infection structures was done according to Koch and associates (1983).

Fig. 3. Restriction of *Phakopsora pachyrhizi* growth on wild-type *Arabidopsis* plants correlated with high frequency of penetrated epidermal cells undergoing cell death. Quantitative assessment of single-cell interaction sites was done for wild-type and mutant *Arabidopsis* plants at 4 dpi. Thereby, seven classes of interaction sites were discriminated, whose frequency is given as the mean plus standard error per leaf. At least four leaves have been analyzed per genotype, and approximately 100 infection sites were inspected per leaf. The first class comprises spores that had germinated but failed to form an appressorium, the second class is represented by those forming an appressorium but without apparent penetration, the next classes describe plant defense reactions, i.e., formation of papilla, trypan-blue staining occurring in penetrated epidermal cells (EPI TB+), the simultaneous staining of epidermal and mesophyll cells (EPI TB+/MESO TB+), and increased hyphal growth in the mesophyll in combination with (EPI TB+/MESO Hyphae) or without (EPI TB-/MESO Hyphae) trypan-blue staining of affected epidermal cells.

Fig. 4. Concomitant knockout of pre- and postpenetration defense did not render *Arabidopsis* a host for Asian soybean rust. Wild-type Col-0, single mutant, or double mutant plants were harvested 2 days after inoculation with *Phakopsora pachyrhizi*. Interaction sites were grouped into different classes comprising sites at which spores had germinated and formed an appressorium and classes in which defense reactions appeared, i.e., formation of papilla, trypan blue staining occurring in penetrated epidermal cells (EPI TB+), staining of epidermal cells in combination with fungal growth in the mesophyll (EPI TB+/MESO TB), and increased hyphal growth in the mesophyll without trypan blue staining of affected epidermal cells (EPI TB-/MESO Hyphae). Frequencies are given as the mean plus standard error per leaf. At least three leaves have been analyzed per genotype, and approximately 30 to 50 infection sites were inspected per leaf.

in the underlying plant tissue (Fig. 4). Obviously, the infection was still developing at 2 dpi, and the frequency of fungal penetration into epidermal cells further increased from 2 (Fig. 4) to 4 dpi (Fig. 3, with interaction sites numbering 15% fewer than found in class 1). On wild-type Col-0 plants, 10% of the attacked epidermal cells countered penetration with papillae and 40% accumulated trypan blue beneath appressoria (Fig. 4). Essentially, microscopic investigation revealed no differences in the frequency of cellular interaction sites between wild-type Col-0 plants and *jar1-1* mutant plants. When compared with Col-0, considerably more pathogen growth was found in mesophyll tissue of the *eds1-2* mutant, and a further increase was found in *sid2-1* plants. Among the single mutants tested in this experiment, *pen3-1* allowed the highest rate of fungal growth in the mesophyll tissue.

Next, we inoculated the double mutants *pen3-1/jar1-1*, *pen3-1/sid2-1*, and *pen3-1/eds1-2* with *P. pachyrhizi*. Among these, the microscopic analysis revealed no substantial differences between *pen3-1/eds1-2* double- and *pen3-1* single-mutant plants (Fig. 4). Since both the *pen3-1* and the *eds1-2* single mutants differ from the wild type but the double mutant did not display an additive effect, it could be concluded that effects of *eds1* and *pen3* mutations affect the same defense pathway. In contrast, the other double mutants showed enhanced colonization of the mesophyll by *P. pachyrhizi* when compared with *pen3-1* plants. Mesophyll colonization could be estimated by adding the frequencies of the last three categories presented in Figure 4. Essentially, 65 and 58% of the interaction sites on the *pen3-1/jar1-1* and *pen3-1/sid2-1* mutant plants, respectively, affected the mesophyll, which was far more than that observed for the *pen3-1* single mutant (26%). Thus, we concluded that *JAR1* and *SID1* act synergistically with *PEN3* in controlling ASR development on *Arabidopsis*. Of all the double mutants tested, the highest frequency of interaction sites with hyphae in the mesophyll but without trypan-blue staining in epidermal cells was found for the *pen3-1/jar1-1* mutant. This may suggest that *JAR1* contributes to cell death induced by *P. pachyrhizi*. Moreover, fungal colonization on this double mutant is speeded up as compared with the other genotypes, as evidenced by a lower frequency of class 1 interaction sites (Fig. 4).

Detection of H₂O₂ in *Arabidopsis* leaves infected with ASR.

To assay the potential involvement of H₂O₂ production in the *Arabidopsis*-ASR interaction, 3,3-diaminobenzidine tetrahydrochloride (DAB) staining was employed. We discriminated three types of DAB-associated plant responses: i) DAB coloration present in penetrated epidermal cells but absent from the mesophyll (Fig. 5A and B), ii) DAB coloration present in penetrated epidermal cells and the mesophyll (Fig. 5C and D), and iii) penetrated epidermal cells without but adjacent mesophyll cells with DAB coloration (Fig. 5E and F). The frequency of each of these categories was monitored on Col-0 or *pen3-1* genotypes at 1 dpi (Fig. 5). Additionally, infection sites were counted that did not show DAB coloration in either the epidermis or in the mesophyll. We also counted infection sites at which penetration attempts resulted in the formation of a papilla. Thus, a total of five categories were measured quantitatively. Clear differences were observed between Col-0 and *pen3-1* plants (Fig. 5). On wild-type Col-0 plants, approximately 50% of the infection sites did not display any DAB coloration, while 40% of the infection sites showed coloration in penetrated epidermal cells. These percentages were very similar to those observed for the corresponding classes after trypan-blue staining (Fig. 3). DAB coloration of mesophyll cells occurred exclusively on *pen3-1* mutants, in which it was associated with enhanced pathogen growth. Interestingly, 20% of the interaction sites showed DAB coloration in the mesophyll without coloration of the adjacent penetrated epi-

Fig. 5. Tissue-specific accumulation of H₂O₂ was correlated with arrest of Asian soybean rust on Col-0 wild-type and *pen3-1* mutant plants. H₂O₂ accumulation was monitored by 3,3-diaminobenzidine tetrahydrochloride (DAB) staining of infected *Arabidopsis* Col-0 and *pen3-1* leaves 24 h postinoculation (hpi) with bright-field microscopy. The upper panel shows six micrographs of infection sites (A-F) in which the focus was either on the epidermal cell layer (A, C, and E) or on the mesophyll cell layer underneath (B, D, and F). A quantitative assessment of H₂O₂ accumulation in inoculated leaves of Col-0 or *pen3-1* is presented in the two diagrams shown in the lower panel. Plant responses to formation of appressoria were classified in different categories: i) no detection of DAB staining (DAB negative), ii) fungal penetration was counterattacked by formation of papillae (papillae), iii) DAB staining solely in epidermal cells (DAB epi.), iv) DAB staining occurring concomitantly in epidermal and mesophyll cells (DAB epi.+meso.) and v) DAB staining only in mesophyll tissue (DAB meso.). Data were collected from 12 leaves from three independent plants per genotype, harvested at 24 hpi. A minimum of 100 interaction sites were inspected per leaf. Frequencies for each category are given as the mean plus standard deviation per leaf.

dermal cells. Again, this was a striking similarity to the results obtained by trypan-blue analysis, in which also a considerable number of penetrated epidermal cells of *Arabidopsis* mutants did not accumulate dye, although the fungus spread into the mesophyll (Figs. 3 and 4).

Transcript accumulation in ASR-infected *Arabidopsis* plants.

The expression profiles of genes encoding for pathogenesis-related 1b (*PR-1b*) protein and *PDF1.2* were investigated in *Arabidopsis* in response to a *P. pachyrhizi* infection (Fig. 6). Neither transcripts for *PR-1b* nor *PDF1.2* could be detected in noninoculated wild-type *Arabidopsis* plants (Fig. 6). *PR-1b* transcripts also did not accumulate after inoculation with *P. pachyrhizi*. By contrast, *PDF1.2* transcripts accumulated by 24 h postinoculation (hpi) with *P. pachyrhizi* and their accumulation decreased during the next 2 days (Fig. 6).

Accumulation of *PR-1b* and *PDF1.2* transcripts upon inoculation with *P. pachyrhizi* was also measured in other *Arabidopsis* mutants (Fig. 6). Basically, noninoculated plants did not show detectable levels of transcripts for the two genes, except *pen3-1*, in which transcripts for both genes were found at 48 hpi. In mutant *pen2-3/pen3-2*, *PR-1b* transcripts accumulated weakly at 1 and 2 days after mock treatment (Fig. 6). Inoculation with *P. pachyrhizi* led to the accumulation of *PDF1.2*

transcripts in all mutants. *PDF1.2* accumulation in infected mutant plants was highest at 24 and 48 hpi and declined subsequently. Strong accumulation of *PR-1b* transcripts was detected in *pen3-1* single- and *pen1-1/pen2-1* double-mutant plants after infection with *P. pachyrhizi*. Accumulation of *PR-1b* mRNA was highest at 72 hpi, and this profile correlated inversely with that observed for the accumulation of *PDF1.2* transcripts (Fig. 6).

Accumulation of transcripts of the *Arabidopsis* gene *NHL10* is considered to be a marker for cells undergoing a hypersensitive response (HR) (Zheng et al. 2004). Here, we tested whether *NHL10* expression was triggered during ASR infection of *Arabidopsis*. Quantitative polymerase chain reaction (qPCR) analysis revealed a clear distinction between the genotypes tested (Fig. 7). Mock-inoculated *pen3-1* mutant plants had accumulated *NHL10* transcripts by 48 h. Significantly more *NHL10* transcripts accumulated in ASR-inoculated *pen3-1* than in the mock-inoculated controls. In contrast, *NHL10* transcripts did not accumulate in mock- or ASR-inoculated Col-0 or *pen2-1/pad4-1* plants.

DISCUSSION

Modern breeding programs for crops include the identification and optimization of effective and durable resistance traits. The so-called NHR response is particularly effective because it provides protection against all isolates of a pathogen species (Heath 2001). Due to this robust protection, NHR is being intensively studied as a source for resistance traits that might help in improving crop performance in the glasshouse and the field (Ellis 2006; Thordal-Christensen 2003).

NHR to rusts is typically mediated prior to formation of the first haustorium (Heath 1981). Our microscopic analyses with

Fig. 6. Timecourse of accumulation of transcripts for *PDF1.2* and *PR-1b* showed an inverse responsiveness to infection with Asian soybean rust. Different *Arabidopsis* genotypes were infected with *Phakopsora pachyrhizi*, and noninfected plants served as a control (mock treatment). Total RNA was extracted from leaves harvested at timepoints indicated. RNA was subjected to RNA gel-blot analysis with gene-specific probes. Equal loading of RNA gels was confirmed after staining with ethidium-bromide (EtBr).

Fig. 7. *NHL10* transcripts accumulated to high levels in *pen3-1* after infection with Asian soybean rust. Leaves infected with *Phakopsora pachyrhizi* (*Pp*) and noninfected leaves (mock) were harvested at timepoints indicated. Relative abundance of *NHL10* transcripts was determined using quantitative polymerase chain reaction and was calculated in relation to the constitutively expressed transcripts of actin2. RNA used in this analysis was from the same preparation as that used for RNA gel-blot analysis. Mean and standard error were calculated as described in Livak and Schmittgen (2001).

Arabidopsis have shown that germination of *P. pachyrhizi* uredospores and the formation of appressoria were unaffected on this nonhost plant. Infection proceeded by formation of penetration hyphae that completely traversed epidermal cells, which concomitantly underwent a cell-death response, as evidenced by trypan-blue staining (Figs. 2 and 3). A similar abrogation of *P. pachyrhizi* development in the plant epidermis has been documented for other nonhost plants (Hoppe and Koch 1989). The authors reported that *P. pachyrhizi* never overcame the epidermal barrier, and it was concluded that collapse of epidermal cells might arrest fungal progress.

The involvement of reactive oxygen species in host-resistance and NHR responses of plants to pathogens has been extensively reviewed (Apel and Hirt 2004; Hückelhoven and Kogel 2003; Mehdy 1994; Nimchuk et al. 2003). Here, we demonstrated that H₂O₂ accumulated in approximately 50% of penetrated epidermal cells of wild-type *Arabidopsis* plants as early as 1 day after inoculation. This frequency further increased to 70% of penetrated cells during the second day of infection (data not shown). Levine and associates (1994) suggested that the accumulated H₂O₂ may orchestrate programmed cell death resulting in HR. This is consistent with our results, which show very similar but slightly time-shifted frequencies of penetrated epidermal cells that showed DAB coloration and trypan-blue staining (Figs. 3 and 5). However, rapid death of attacked epidermal cells may not lead to the complete arrest of fungal colonization, because this epidermal defense response also occurs on the susceptible soybean host (Koch et al. 1983). Hence, it seems that either the relative rates of initiation of epidermal HR or one or more unknown contributions of the mesophyll tissue might be crucial for the effectiveness of NHR in the *Arabidopsis*-*P. pachyrhizi* interaction. The latter seems more likely, because arrest of the fungus was manifested after it traversed the epidermal tissue and started to enter into the mesophyll (Fig. 2).

Since *P. pachyrhizi* is a direct-penetrating rust, we included *Arabidopsis* mutants with defects in epidermal defense in our studies. The *pen1*, *pen2*, and *pen3* genotypes have been reported to allow enhanced entry of nonadapted powdery mildew pathogens into the cell periphery (Collins et al. 2003; Lipka et al. 2005; Stein et al. 2006). As shown in this study, the three mutations led to enhanced invasive growth of *P. pachyrhizi* into the mesophyll (Fig. 3). On the basis of our results, it seems likely that there is an interplay of the respective wild-type alleles during the arrest of *P. pachyrhizi* in the epidermis of wild-type *Arabidopsis*. Among the *pen*-mutant plants we tested, the highest rate of mesophyll colonization was found on *pen3-1*, and cell death in the mesophyll was observed frequently. Mesophyll cell death also was associated with the resistance of a particular soybean host variety to ASR (Koch et al. 1983). However, this variety was not successful in the field. It was suggested that mesophyll cell death, most likely being initiated upon direct contact with haustorial mother cells or during haustorium initiation, presented the final barrier before irreversible establishment of disease (Hoppe and Koch 1989). Therefore, we conclude that a presumably first line of (non-host) defense in wild-type *Arabidopsis* is shifted, due to the *pen3-1* mutation, to the very last line of (host) defense. Similarly to the wild type, the majority of penetrated epidermal cells in the *pen1-1* and *pen2-1* mutants underwent cell death (Fig. 3). However, in approximately half of these incidences, an increased rate of fungal hyphal penetration into the mesophyll was observed, as compared with the wild type. The *pen1-1* mutation showed a slightly lesser effect in this regard than the *pen2-1* mutant. This observation might be due to the fact that *PEN1* has the highest impact on NHR against nonadapted powdery mildews, while *PEN2* and *PEN3* also control

entry of other nonhost pathogens, as shown for *Phytophthora infestans* and *Plectosphaerella cucumerina* (Lipka et al. 2005; Stein et al. 2006).

On mutant *Arabidopsis* plants, a few interaction sites were found in which epidermal cell death was lacking even though fungal hyphae grew into the mesophyll (Figs. 3 and 4). It appears that cell death may have a dual role in interactions of potential hosts with *P. pachyrhizi*. Thus, it was suggested that ASR needs epidermal cell death to permit colonization of its soybean host (Koch et al. 1983), and if it is further assumed that epidermal cell death may contribute to the resistance of nonhost plants against ASR, it could be speculated that penetration and development in the mutant plants proceeded rapidly under these circumstances before epidermal cell death was triggered.

In the nonhost interaction between *Arabidopsis* and *Erysiphe pisi*, the simultaneous loss of two barriers conditioned by the *PEN1* and *PEN2* genes, namely resistance to penetration and postpenetration resistance, respectively, was sufficient to allow growth and asexual reproduction of the nonhost pathogen (Lipka et al. 2005). Similarly, in the work reported here, inoculation of the *pen2-1/pad4-1* double mutant with *P. pachyrhizi* resulted in enhanced colonization by the pathogen when compared with the *pen2-1* single mutant (Fig. 3). It could be speculated that the simultaneous knock-out of these two levels of resistance speeds up mesophyll colonization, which finally permits the formation of haustoria-like structures (Fig. 2H). However, the pathogen did not sporulate at these infection sites. Therefore, it must be concluded that establishment of a successful biotrophic interaction failed. To further analyze components involved in this block of compatibility, we took advantage of mutants with defects in SA or JA signaling. These two signal transduction pathways are known to contribute to resistance against biotrophic and necrotrophic pathogens, respectively (Glazebrook 2005; Thaler et al. 2004). Since it is clear that the effects of the postpenetration line of defense can only get visible after diminishing the penetration resistance, we conducted these experiments in a *pen3-1* genetic background, i.e., using the double mutants *pen3-1/jar1-1*, *pen3-1/sid2-1*, and *pen3-1/eds1-2* (Fig. 4). Microscopic investigation suggested that the effect of *EDS1* on ASR development is coupled to *PEN3*, while *JAR1* and *SID2* seemed to act synergistically with *PEN3* (Fig. 4). Since the effect of *JAR1* becomes visible only in a *pen3-1* genetic background, it could be speculated that *JAR1* might be involved in postpenetration resistance. Nevertheless, neither the *pen2-1/pad4-1* nor any of the *pen3-1* double mutants supported growth of large fungal colonies or sporulation. This finding indicates a role for further, as yet unidentified, genes in controlling the NHR of *Arabidopsis* against *P. pachyrhizi*. Alternatively, it could be that essential requirements that are needed to establish the biotrophic stage of the pathogen are missing in *Arabidopsis*.

Arabidopsis-pathogen interactions are characterized by activation of distinct gene sets. Expression of the gene encoding PR-1b protein is widely thought to be a molecular marker for SA-mediated defense, while transcripts for plant defensin (*PDF1.2*) accumulate strongly in JA-mediated resistance against necrotrophs (Glazebrook 2001). RNA gel-blot analyses revealed a strong accumulation of *PDF1.2* transcripts in *Arabidopsis* mutants in comparison with the wild type after inoculation with *P. pachyrhizi*. Strikingly, transcript accumulation peaked coincidentally with death of penetrated epidermal cells (Figs. 3 and 6). This finding was somewhat unexpected, because the plant defensin *PDF1.2* was previously shown to accumulate in leaves of *Arabidopsis* upon challenge with necrotrophic fungi as a part of the JA- and ethylene-dependent defense mechanism (Glazebrook 2005). The fact that the osten-

sibly obligate biotrophic fungus *P. pachyrhizi* triggered components of the JA pathway raised questions about whether this pathogen may, at least in parts of its life cycle, mimic a necrotroph. If this were correct, it could be speculated that *P. pachyrhizi* is able to survive in epidermal cells undergoing a cell-death response (discussed above), which has been proposed as a characteristic of necrotrophs (Mayer et al. 2001). However, the correlation of *PDF1.2* expression with infections by necrotrophs seems not to be absolute, since transcripts also accumulated in *Arabidopsis* in response to infection with the nonadapted biotrophic barley powdery mildew fungus (Yun et al. 2003) but, interestingly, not after infection with the wheat powdery mildew fungus (Zimmerli et al. 2004). Thus, it seems that particular defense responses that are triggered during NHR do not depend on the (biotrophic or necrotrophic) lifestyle of the challenging pathogen but, rather, are a distinct response to a particular pathogen species. Indeed, there is evidence for specific recognition events in NHR to pathogens (Heath 2001; Matsumura and Tosa 1995).

In contrast to *PDF1.2*, expression of *PR-1b*, a reliable marker for activation of the SA-dependent resistance pathway, which is primarily involved in resistance to biotrophs, was detected at later stages of *P. pachyrhizi* infection in *Arabidopsis* mutants. *PR-1b* transcript accumulation occurred concomitantly with colonization of mesophyll tissue, an unquestionably biotrophic aspect of ASR etiology. Levels of JA and SA affect each other inversely (Spoel et al. 2003). Thus, an infection strategy that initially camouflages ASR's biotrophic lifestyle and does not trigger SA signaling might have advantages in establishing disease. This hypothesis was further supported by the finding that *sid2-1* mutants, which are impaired in SA-mediated resistance, were more frequently penetrated by *P. pachyrhizi* (Fig. 4). It could be hypothesized that the transcript profiles observed point to a widening of the pathogen's lifestyle, i.e., adopting or maintaining properties of necrotrophy, which might be one reason leading to a broader host range.

Mutations in the *NDR1* gene (non-race specific disease resistance 1) renders *Arabidopsis* susceptible to several strains of the bacterium *Pseudomonas syringae* pv. *tomato* and to the oomycete pathogen *Hyaloperonospora parasitica* (Century et al. 1995). *NDR1* shares homology to the tobacco *HIN1* (harpin-induced 1) gene, which was found to be up-regulated during the HR (Lee et al. 2001; Takahashi et al. 2004). However, among the 45 *Arabidopsis* genes that show sequence similarity to tobacco *HIN1*, only *NHL10* (*NDR1/HIN1*-like) but not *NDR1* behaved like the tobacco gene by being up-regulated during the HR and in senescing leaves (Zheng et al. 2004). Interestingly, transcripts for *NHL10* accumulated to high levels in *Arabidopsis* leaves of the *pen3-1* mutant upon challenge with *P. pachyrhizi* (Fig. 7). Since expression of the gene, which could be considered as a potential marker for *Arabidopsis* tissue undergoing HR (Zheng et al. 2004), was neither triggered in wild-type *Arabidopsis* nor in the *pen2-1/pad4-1* mutant upon ASR inoculation, it must be concluded that either the cell-death responses in these genotypes differ from those observed in *pen3-1* or that signals that are required for *NHL10* expression were negatively influenced by wild-type *PEN3*.

Our results differ from those reported in another recent study of the NHR of *Arabidopsis* against a nonadapted rust fungus from grasses (*Puccinia triticina*) (Shafiei et al. 2007). The authors tested a series of *Arabidopsis* mutants with defects in defense signaling, including single mutants tested in the present study, but none of the mutants tested displayed higher susceptibility to *Puccinia triticina*. This contrasts with a report on the infection of *Arabidopsis* with a nonhost dicot rust (*Uromyces vignae*) (Mellersh and Heath 2003). This pathogen was able to establish large fungal colonies on *Arabidopsis* mutants

that were compromised in SA signaling. The differences between the two studies on stoma-penetrating rusts and our work presented here with the directly penetrating ASR made it obvious that NHR of *Arabidopsis* against rusts does not rely on a single mechanism. The same conclusion has been drawn before by Mellersh and Heath (2003). Moreover, our study demonstrated that the contribution of SA and JA in NHR cannot be predicted for a given fungal pathogen from its lifestyle without further investigation. Further studies will reveal whether the observed specificity in the NHR of *Arabidopsis* against *P. pachyrhizi* might lead to new concepts in our understanding of NHR.

MATERIALS AND METHODS

Fungal and plant material.

The isolate of *P. pachyrhizi* was taken from infected plant material collected in Brazil. The fungus was maintained on the susceptible soybean cultivar Pintada. Soybean seeds were germinated for 2 to 3 days in petri dishes at room temperature in the dark and were transplanted to a 2:1 mixture of soil (P-substrate, Balster Einheitserde GmbH, Froendenberg, Germany) and sand. Plants were grown in a growth chamber at 22°C, 60% relative humidity, and a photoperiod of 16 h at 133 $\mu\text{mol m}^{-2} \text{s}^{-1}$.

Arabidopsis seeds from wild-type Col-0 and mutant plants Col-3 *gl1* (mutation in *GLABROUS 1*), *pen1-1*, *pen2-1*, *pen3-1*, and double mutants *pen1-1/pen2-1*, *pen2-3/pen3-2*, and *pen2-1/pad4-1* (Collins et al. 2003; Lipka et al. 2005; Stein et al. 2006) were provided by V. Lipka, Sainsbury Laboratory, Norwich, U.K. Double mutants *pen3-1/jar1-1*, *pen3-1/sid2-1*, *pen3-1/pad4-1*, and *pen3-1/eds1-2* were provided by P. Schulze-Lefert, Max Planck Institute for Plant Breeding Research, Cologne, Germany. Plants were cultivated in a growth chamber at short-day conditions. *Arabidopsis* accessions tested in response to *P. pachyrhizi* inoculation were as follows: Ag-0, Ak, Bay-0, Br-0, Bur-0, Can-0, Ct-1, Cvi, Cvi-0, Do-0, Eri, Fei-0, Ga-0, Gy-0, Kin-0, Kondara, Ll-0, Mt-0, Mz-0, Nd-1, Nok-3, Sha, Sorbo, Ts-5, Tsu-0, Van-0, Ws-0, Wt-5.

Uredospore suspensions for inoculation were generated by washing an infected primary soybean leaf in 50 ml of 0.1% (vol/vol) Tween-20 in distilled water. After foliar spray application onto primary leaves of 16-day-old plants, these were placed in a dark moist chamber (26°C and nearly 100% relative humidity) for 24 h. Afterwards, the plants were kept under growth conditions. Rust pustules were visible on infected plant material approximately 10 days after inoculation. Infected leaves were used as a source for inoculation after another 4 days. Fresh uredospores for inoculation were generated by removing old uredospores from infected leaves by shaking 1 day before spore harvest.

Microscopic analysis.

Infected leaves were harvested at the timepoints indicated and were stained with trypan blue (Keogh et al. 1980). After cutting, leaves were directly submerged in ethanolic lactophenol-trypan blue staining solution and were heated to 90°C for 3 min. Leaf samples were cooled to room temperature for 10 min and were subsequently incubated in saturated chloral hydrate (2.5 g ml^{-1}) for a couple of days. During that time, chloral hydrate was changed several times, until leaves did not release blue stain anymore. Leaves were stored in 25% (vol/vol) glycerol. Fungal structures were inspected by Bright-field microscopy using a Leica DMRBE microscope (Leica, Bensheim, Germany). Images were taken with a digital JVC KYF 750 camera. Three to five leaves were analyzed for each genotype, and a total of 80 to 150 infection sites were inspected per leaf and timepoint.

DAB staining.

H₂O₂ was visualized by applying a modified protocol of Thordal-Christensen and associates (1997). Leaves were harvested 24 hpi and were incubated in DAB solution (1.68 mg ml⁻¹, pH 3.8) for 8 h under high humidity. During the incubation period, only the leaf petiole was in contact with the DAB solution. Therefore, the uptake of stain was driven by transpiration flow. Subsequently, leaves were fixed and cleared in saturated chloral hydrate (2.5 g ml⁻¹), were mounted in 50% (vol/vol) glycerol, and were examined by bright-field microscopy. Vacuum infiltration of DAB and 10 mM H₂O₂ was done as a control and confirmed equal distribution of peroxidases within leaves. Specificity of the staining was further confirmed by an enrichment of the DAB solution with 50 mM ascorbic acid, which entirely inhibited accumulation of brown DAB precipitate in the presence of 10 mM H₂O₂ (data not shown).

RNA gel-blot analyses.

Total RNA was extracted using the acid guanidinium thiocyanate-phenol-chloroform protocol (Chomczynski and Sacchi 1987). For gel-blot analyses, 10 µg of total RNA were separated on 1.5% (vol/vol) formaldehyde agarose gels (Sambrook et al. 1989), were transferred to GeneScreen membranes, and were cross-linked using UV light (Amersham, Little Chalfont, U.K.). Equal gel loading was monitored by ethidium-bromide staining of the gel before RNA transfer. Membranes were hybridized according to Peterhänsel and associates (1998) in Dig Easy Hyb (Roche, Mannheim, Germany) at 65°C overnight with a fluorescein- or digoxigenin-labeled probe that was generated by *in vitro* transcription. After a stringent wash step at 60°C with 0.1 × SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 0.1% sodium dodecyl sulfate (SDS), immunodetection of probes was done in combination with chemiluminescence detection using anti-fluorescein-Fab or antidigoxigenin-Fab fragments and CSPD (Roche, Mannheim, Germany). cDNAs used as the probes are designated *PR1b* (At2g14610) and *PDF1.2* (At5g44420).

qPCR.

RNA (1.25 µg) was incubated with 1 U of DNaseI (Fermentas GmBH, St. Leon-Rot, Germany) for 30 min at 37°C, followed by a 15-min enzyme inactivation step at 70°C. OligodT primer (HindANCHORT 5'-AAG CTT TTT TTT TTT TTT T(AGC)-3') (1 pmol) was added, and the reaction mix was treated for 5 min at 70°C. Reverse transcription was conducted for 1 h at 37°C, using 100 U of reverse transcriptase (Revert Aid M-MuLV, Fermentas GmbH). After enzyme inactivation for 10 min at 70°C, first-strand cDNA synthesis was completed.

qPCR was conducted using an ABI PRISM 7000 sequence detector (Applied Biosystems, Darmstadt, Germany) and SYBR Green master mix (Invitrogen, Karlsruhe, Germany). First-strand cDNA reaction mix was diluted 1:20, and 2 µl was subjected to PCR using *AtNHL10*-gene specific primers (1479F 5'-AAC GTT CCA AGG CCA AAA CC-3', 1531R 5'-AAG TCC TAG ACT GTC CGG CGT-3') at a concentration of 0.5 µM each. PCR-cycling conditions were implemented as follows: 2 min 50°C, 10 min 95°C, followed by 40 repetitions of 15 s at 95°C, and 1 min at 60°C. Expression of actin was followed using gene-specific primers (2F 5'-GCT AAC ATT GTG CTC AGT GGT GG-3', 2R 5'-GGT GCA ACG ACC TTA ATC TTC AT-3') and served as an internal standard. The ABI PRISM 7000 SDS 1.0 program (Applied Biosystems) was used to analyze the results. The mean and standard deviation for the relative abundance of *AtNHL10*-specific transcripts were calculated according to the mathematical model of Livak and Schmittgen (2001).

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