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Arabidopsis PEN3/PDR8, an ATP Binding Cassette Transporter, Contributes to Nonhost Resistance to Inappropriate Pathogens That Enter by Direct Penetration [W] [OA]

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***Arabidopsis thaliana* is a host to the powdery mildew *Erysiphe cichoracearum* and nonhost to *Blumeria graminis* f. sp *hordei*, the powdery mildew pathogenic on barley (*Hordeum vulgare*). Screening for *Arabidopsis* mutants deficient in resistance to barley powdery mildew identified *PENETRATION3* (*PEN3*). *pen3* plants permitted both increased invasion into epidermal cells and initiation of hyphae by *B. g. hordei*, suggesting that PEN3 contributes to defenses at the cell wall and intracellularly. *pen3* mutants were compromised in resistance to the necrotroph *Plectosphaerella cucumerina* and to two additional inappropriate biotrophs, pea powdery mildew (*Erysiphe pisi*) and potato late blight (*Phytophthora infestans*). Unexpectedly, *pen3* mutants were resistant to *E. cichoracearum*. This resistance was salicylic acid–dependent and correlated with chlorotic patches. Consistent with this observation, salicylic acid pathway genes were hyperinduced in *pen3* relative to the wild type. The phenotypes conferred by *pen3* result from the loss of function of PLEIOTROPIC DRUG RESISTANCE8 (PDR8), a highly expressed putative ATP binding cassette transporter. PEN3/PDR8 tagged with green fluorescent protein localized to the plasma membrane in uninfected cells. In infected leaves, the protein concentrated at infection sites. PEN3/PDR8 may be involved in exporting toxic materials to attempted invasion sites, and intracellular accumulation of these toxins in *pen3* may secondarily activate the salicylic acid pathway.**

INTRODUCTION

Nonhost resistance is the type of nonspecific resistance that an entire plant species exhibits against all genotypes within a pathogen species (Thordal-Christensen, 2003). Although until recently little was known about the biochemical defenses that contribute to nonhost resistance, cytological descriptions suggested that a majority of inappropriate fungal pathogens were unable to breach the plant cell wall and infiltrate host cells (Yun et al., 2003; Zimmerli et al., 2004). However, some variation in penetration efficiency exists among different host and fungal pathogen combinations (Mellersh and Heath, 2003). These studies also showed that plants respond actively to attack by inappropriate pathogens and do not rely solely on preformed and constitutive barriers for protection against inappropriate pathogens (Meyer and Heath, 1988; Zimmerli et al., 2004). Furthermore, it appears that operationally, nonhost resistance can be divided into penetration resistance, barriers limiting entry of the pathogen into cells, and postpenetration resistance, mechanisms that act intracellularly if penetration resistance is overcome (Fernandez and Heath, 1991; Huitema et al., 2003; Mellersh and Heath, 2003; Yun et al., 2003; Zimmerli et al., 2004; Lipka et al., 2005).

Both surveys of mutants with defects in various defense functions and screens for mutants specifically compromised in nonhost resistance have identified a diverse group of genes that contribute to nonhost resistance. Among the mutants with defects in the salicylic acid (SA) signal transduction pathway, nonhost resistance was diminished in *eds1* mutants and transgenic plants with the bacterial *NahG* gene relative to wild-type plants (Parker et al., 1996; Mellersh and Heath, 2003; Yun et al., 2003; Zimmerli et al., 2004). Using virus-induced gene silencing, the chaperone and chaperone-like proteins HSP70, HSP90, and SGT1, which are thought to stabilize resistance (R) proteins and act upstream of EDS1, were shown to contribute to nonhost resistance in *Nicotiana benthamiana* (Peart et al., 2002; Kanzaki et al., 2003). Mutants with defects in the ethylene/jasmonate (ET/JA) signal transduction pathways exhibited wild-type levels of nonhost resistance in *Arabidopsis thaliana*, even though there is evidence from microarray studies that this pathway is preferentially induced after inoculation of plants with some inappropriate pathogens (Huitema et al., 2003; Zimmerli et al., 2004). These data suggest that the ET/JA pathway and components of the SA

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pathway acting downstream of *eds1* play no role or only a limited role in nonhost resistance. Alternatively, it is possible that these pathways contribute to resistance to inappropriate pathogens only after the other defenses have been defeated (Jones and Takemoto, 2004). Because EDS1 contributes to basal resistance (Parker et al., 1996), defenses that act to limit the growth of appropriate pathogens even in compatible interactions, it seems likely that there is some overlap between basal resistance and nonhost resistance.

Additional host components, for which no clear role in basal resistance has been demonstrated to date, contribute to nonhost resistance. NHO1, a glycerol kinase, is required for nonhost resistance in *Arabidopsis* to a bacterial pathogen of bean (*Phaseolus vulgaris*), *Pseudomonas syringae* pv *phaseolicola* (Kang et al., 2003). In physiological studies using inhibitors, the actin cytoskeleton was shown to be required for nonhost resistance against barley (*Hordeum vulgare*) and wheat (*Triticum aestivum*) powdery mildews in pea (*Pisum sativum*) and *Arabidopsis*, respectively (Kobayashi et al., 1997; Yun et al., 2003). These genes do not fit into a single pathway or process, but their identification does suggest that diverse processes contribute to nonhost resistance. This is consistent with an early model of nonhost resistance in which it was proposed that this form of resistance consists of several barriers operating in parallel to limit pathogen colonization (Heath, 2000).

To identify the components required for nonhost resistance, we screened for *Arabidopsis* mutants allowing increased penetration by the barley powdery mildew *Blumeria graminis* f. sp *hordei*, assuming that such mutants would carry defects in those components of nonhost resistance that limit pathogen entry into host cells. The syntaxin *PEN1* (=*SYP121*) and the glycosyl hydrolase *PEN2* were recovered from this and related screens (Collins et al., 2003; Lipka et al., 2005). Here, we describe a third mutant isolated from this screen and show that *PEN3* encodes the putative ATP binding cassette (ABC) transporter PDR8. Subcellular localization of PEN3 in the plasma membrane and extensive gene interaction studies lead us to speculate that PEN3 mediates the targeted export of toxins to penetration sites.

RESULTS

pen3 Lacks Penetration Resistance to Three Inappropriate Pathogens

We conducted a screen for the loss of penetration resistance by screening for *Arabidopsis* mutants that allowed *B. g. hordei* to form haustoria within epidermal cells at a higher frequency than on wild-type plants. Haustoria, which are fungal feeding structures encased in a specialized host membrane within epidermal cells, are produced once the fungal germlings have successfully breached the host cell wall. The few *B. g. hordei* haustoria that formed in *Arabidopsis* were rapidly encased in callose and could be recognized by their distinctive oval shape in inoculated leaves stained with the callose stain aniline blue (Figure 1E, inset). Nine *pen* mutants were recovered from ~12,000 M2 plants from ethyl methanesulfonate–mutagenized seeds. Mapping experiments and crosses among the mutants were used to place the *pen* mutants in complementation groups. When the identities of the *PEN* genes became known, allelism among the various mutants was confirmed by sequencing the appropriate gene (i.e., *PEN1*, *PEN2*, or *PEN3*). *pen1-4* (Collins et al., 2003), *pen2-3* (Lipka et al., 2005), *pen3-1*, and *pen3-2* were recovered from this screen. *pen3-1*, like the *pen1* and *pen2* mutants, supported a higher frequency of fungal penetration (Figure 1A). Another feature of the phenotype conferred by *pen3* was that callose deposition lining the entire periphery of invaded epidermal cells was more common than in wild-type plants (Figures 1D and 1E). Compared with wild-type plants, *pen3-1* plants also allowed increased frequency of formation of elongating secondary hyphae, an indication that the underlying haustoria were functional (Figures 1A to 1C). Secondary hyphae are the hyphae that emerge from the conidium after the first haustorium is established. Germ tube growth is supported by reserves in the conidium, whereas the emergence and growth of secondary hyphae are thought to be dependent on nutrients acquired from the host via the haustorium (Masri and Ellingboe, 1966; Ellingboe, 1972). Therefore, *pen3-1* mutants were partially compromised not only in penetration resistance against *B. g. hordei* but also in a mechanism restricting haustorium function. Loss of PEN3 function does not allow *B. g. hordei* to complete its life cycle and form asexual conidia on *Arabidopsis* as it does on barley, suggesting that additional factors contribute to nonhost resistance to *B. g. hordei* in *Arabidopsis*.

To determine whether increased pathogen entry was limited to interactions with *B. g. hordei*, *pen3-1* plants were inoculated with two other inappropriate pathogens. *Erysiphe pisi* (tribe Erysipheae), the powdery mildew pathogenic on pea, belongs to a tribe distinct from *Erysiphe cichoracearum* (tribe Golovinomyceteae) and *B. g. hordei* (tribe Blumerieae) (Saenz and Taylor, 1999; Braun et al., 2002). Similar to *B. g. hordei*, *E. pisi* was able to initiate the formation of elongating secondary hyphae at a higher frequency on *pen3-1* than on wild-type plants (Figure 5D). Likewise, *Phytophthora infestans*, which is a hemibiotrophic oomycete pathogen and the causal agent of late blight in potato (*Solanum tuberosum*), formed invasive hyphae, which were encased in callose (Figure 1I, inset), more often in *pen3-1* mutants than in wild-type plants (Figure 1H). Inoculation with *P. infestans* elicited little callose deposition in wild-type plants, whereas dramatic callose deposition was observed in *pen3-1* epidermal cells (Figures 1H and 1I). When it occurred, penetration by *P. infestans* into *Arabidopsis* epidermal cells was often accompanied by local cell death at sites of infection (Huitema et al., 2003). Although the wild type appeared healthy where droplets of *P. infestans* zoospores had been applied (Figure 1F), *pen3-1* exhibited macroscopic cell death (Figure 1G), consistent with the idea that a greater proportion of oomycete individuals had penetrated into host cells and elicited cell death. Thus, *pen3-1* had a diminished capacity to restrict pathogen entry by three different inappropriate pathogens.

PEN3 Encodes a PDR-Like ABC Transporter

Mapping populations were generated by crossing *pen3-1* and *pen3-2* to Landsberg *erecta* (L*er*). Mutant plants identified in the F2 generation were used for bulk segregant mapping (Lukowitz

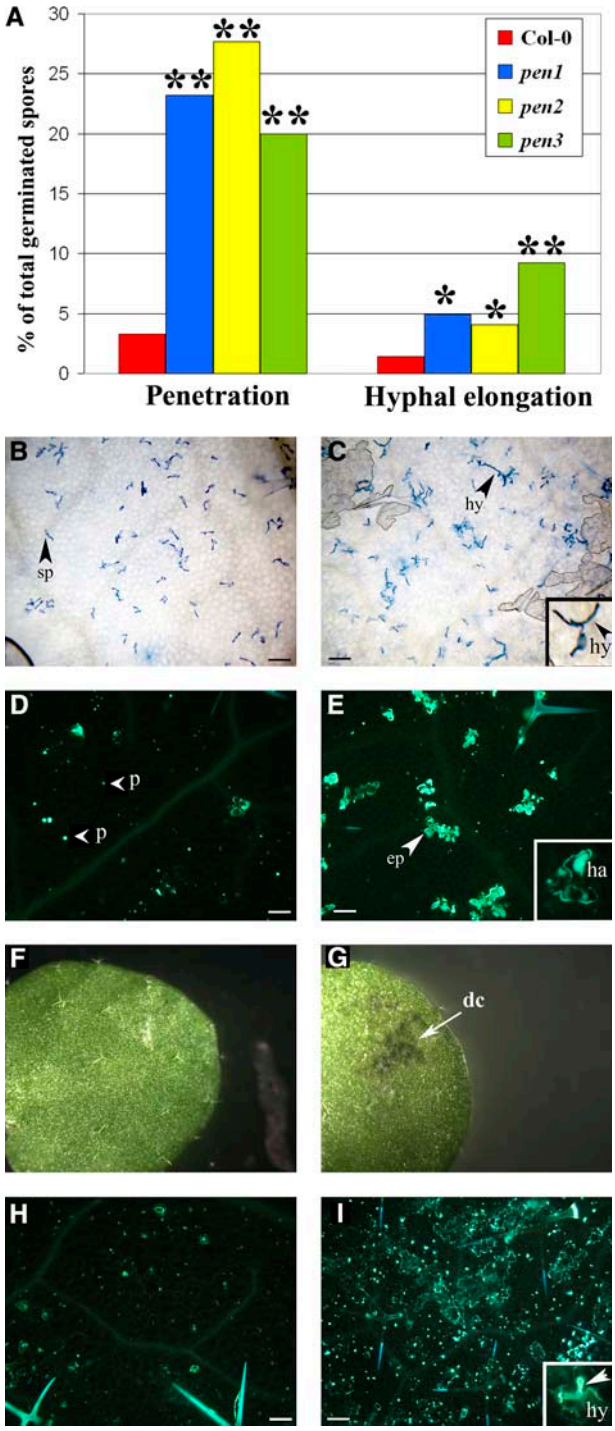


Figure 1. *pen3* Nonhost Resistance Phenotypes.

(A) Mean of the frequency of *B. g. hordei* penetration (left) and hyphal elongation (right) on *Arabidopsis* at 2 DAI, expressed as a percentage of total germinated spores. Wild-type controls are represented in red, *pen1-4* plants in blue, *pen2-3* plants in yellow, and *pen3-1* plants in green. Asterisks denote statistically significant differences between mutants and the wild type by Student's *t* test (** $P < 0.0001$, * $P < 0.001$).

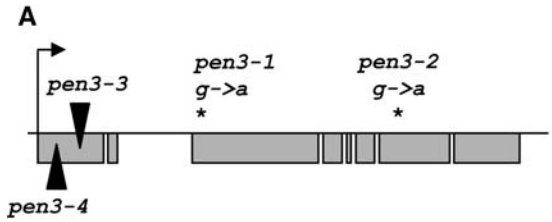
(B) and **(D)** *B. g. hordei* inoculation on Columbia-0 (Col-0). Conidia (sp; arrowhead) germinate and produce appressoria but seldom are able to penetrate and establish functional haustoria **(B)**. Papillae (p; arrowhead), callose-rich cell wall appositions, form at penetration sites whether or not the powdery mildew pathogen is able to successfully breach the cell wall and form a haustorium **(D)**. Papillae were used as markers for attempted penetration sites.

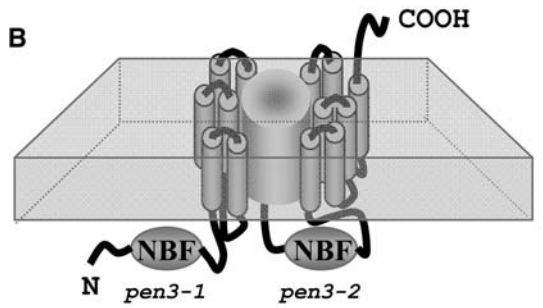
(C) and **(E)** *B. g. hordei* inoculation on *pen3-1*. A higher proportion of conidia are able to penetrate, establish haustoria, and form elongating secondary hyphae (hy) on *pen3* plants relative to Col-0 (**[C]** and inset). Haustorial formation (ha) is associated with callose deposition, encompassing the entire invaded host epidermal cell (ep; arrowhead) (**[E]** and inset).

(F) to **(I)** Response to *P. infestans* inoculation. Cell death (dc; arrow) is evident macroscopically after inoculation of *pen3* **(G)** but not in Col-0 **(F)**. A small amount of callose is deposited in Col-0 **(H)**, whereas widespread callose deposition and occasional intracellular hyphae (hy; arrowhead) are observed in *pen3* (**[I]** and inset).

In **(B)** and **(C)**, samples were stained with trypan blue at 2 DAI and visualized by bright-field microscopy. In **(D)**, **(E)**, **(H)**, and **(I)**, samples were stained with aniline blue at 2 DAI to detect callose and visualized by fluorescence microscopy. Bars = 30 μm.

et al., 2000). In both crosses, *PEN3* mapped to the bottom of chromosome 1 between markers nga280 and ciw1. These two markers were used to identify additional recombinants in this genetic interval from ~3600 F2 plants. In this manner, the interval containing *PEN3* was delimited to a region encompassed by BAC F23H11. Nineteen T-DNA mutant populations with insertions in 1 of 15 genes in this interval were screened for the phenotype conferred by *pen* after inoculation with *B. g. hordei* (Ecker, 2002). Two of these T-DNA populations (SALK__110926 and SALK_000578) segregated plants with a penetration-deficient phenotype. These mutants had predicted insertions in a PDR-like ABC transporter (At1g59870). Both *pen3-1* and *pen3-2* carried single nucleotide substitutions at nucleotides 1419 and 3335, respectively, from the A of the ATG initiation codon of the genomic sequence for this gene (Figure 2A). Two T-DNA alleles, *pen3-3* (SALK_110926) and *pen3-4* (SALK_000578), had insertions near the 5′ end of the coding region (Figure 2A). The *PEN3* gene had previously been annotated as *PDR8* based on its homology with yeast PDR transporters (van den Brule and Smart, 2002). It encodes a 4.3-kb cDNA and is predicted to encode a 1469–amino acid protein with 13 transmembrane domains (Figure 2B). *Arabidopsis* has 15 PDR family members, which are highly similar at the amino acid level (van den Brule and Smart, 2002). An alignment of these members showed that the point mutations in *pen3-1* and *pen3-2* occurred in highly conserved regions of the nucleotide binding folds (Figure 2C). The nucleotide binding folds are thought to mediate ATP binding and consist of a Walker A motif followed by an ABC signature motif and a Walker B motif (van den Brule and Smart, 2002). The mutation in *pen3-1* converted a Gly to an Asp in the ABC signature motif of the first nucleotide binding fold, whereas the mutation in *pen3-2* converted a Gly to a Ser in the Walker A motif of the second nucleotide binding fold. *PEN3* transcript was detected in *pen3-1* and *pen3-2* but not in the T-DNA mutants *pen3-3* and *pen3-4* (data not shown). Given that all four *pen3* alleles have identical phenotypes, we assume that proteins encoded by *pen3-1* and *pen3-2* are nonfunctional, at least in nonhost resistance.





C

PDR8	GISGGQKKRVTT**G**EMIVG	**ABC Signature**
PDR7	GISGGQKKRVTTGEMIVG	ISGGQKKRVTT**G**
PDR1	GISGGQKKRVTTGEMIVG	
PDR2	GISGGEKRRLTTGELVVG	*pen3-1*
PDR3	GVSGGQRKRVTTGEMTVG	**G**354->D
PDR4	GISGGQKKRLTTGELLVG	
PDR5	GISGGQKKRLTTAEMIVG	
PDR6	GISGGQRKRLTTGEMLVG	
PDR9	GISGGQKKRVTTGEMLVG	
PDR10	GISGGQKKRLTTGEMLVG	
PDR11	GISGGQKKRLTTAEMIVG	
PDR12	GISGGQKKRVTTGEMLVG	
PDR13	GISGGQKRRLTT------	
PDR14	GISGGQKRRLTTGEMIVG	
PDR15	GISGGQKRRLTTGEMIVG	
Nt_PDR1	GISGGQKKRVTTGEMLVG	

PDR8	PGVLTALMGVS**G**AGKTTLMDVL	**Walker A Motif**
PDR7	PGVLTALMGVSGAGKTTLMDVL	GVS**G**AGKT
PDR1	PGVLTALMGVSGAGKTTLMDVL	
PDR2	PGVLTSLMGVSGAGKTTLLDVL	
PDR3	PGVLTALVGSSGAGKTTLMDVL	*pen3-2*
PDR4	PGVLTALVGVSGAGKTTLMDVL	**G**915->S
PDR5	PGVLTALMGISGAGKTTLLDVL	
PDR6	PGVLTALVGVSGAGKTTLMDVL	
PDR9	PGVLTALMGVSGAGKTTLMDVL	
PDR10	PGILTALVGVSGAGKTTLMDVL	
PDR11	PGILTALMGVSGAGKTTLLDVL	
PDR12	PGVLTALMGVSGAGKTTLMDVL	
PDR13	PGVLTSLMGVSGAGKTTLLDVL	
PDR14	PGVLTALMGVSGAGKTTLLDVL	
PDR15	PGVLTALMGVSGAGKTTLLDVL	
Nt_PDR1	PGVLTALMGVSGAGKTTLMDVL	

Figure 2. Molecular Identity of *PEN3* and of the *pen3* Alleles.

(A) *PEN3* gene structure indicating exons (gray boxes) and introns (black lines connecting exons). The genomic sequence of *PEN3* is 6.1 kb. Sequencing of the *pen3-1* and *pen3-2* alleles revealed single nucleotide changes (asterisks) that result in amino acid substitutions. Two T-DNA alleles (black arrowheads) carry insertions early in the gene.

(B) Predicted architecture of the PEN3 protein. PEN3 is predicted to have 1469 amino acids. The amino acid changes in *pen3-1* and *pen3-2* occur in the ABC signature motif and the Walker A motif of the first and second nucleotide binding folds (NBF), respectively.

(C) Alignment of amino acids of the two nucleotide binding folds of the 15 members of the *Arabidopsis* PDR family and of tobacco PDR1. Mutations in *pen3-1* and *pen3-2* result in changing highly conserved Gly (boldface, underlined G) to larger amino acids, Asp and Ser, respectively. Both changes occur in pockets thought to mediate ATP binding.

The *Arabidopsis* PDR family of ABC transporters has been studied via RNA gel blotting and RT-PCR to determine in which plant organs these transporters are expressed (van den Brule and Smart, 2002). In addition, publicly available databases provide microarray expression profiles of different *Arabidopsis* organs, tissues, and environmental treatments (Rhee et al., 2003; Zimmermann et al., 2004). These resources showed that *PEN3* was expressed in all tissues, but most highly in leaves. Most other PDR family members were expressed at low levels and in specific plant organs (see Supplemental Table 1 online) (Rhee et al., 2003). In leaves, *PEN3* transcript accumulated to levels comparable to those of metabolic housekeeping genes, such as cytosolic glyceraldehyde-3-phosphate, and 1 to 2 orders of magnitude higher than the other PDR family members (see Supplemental Table 1 online) (Shih et al., 1991). The gene encoding PDR7, the ABC transporter most similar to PEN3 in sequence (90% amino acid similarity), was expressed primarily in roots (see Supplemental Table 1 online), and T-DNA insertion mutations in *PDR7* (SALK_134725 and SALK_008761) did not exhibit the penetration-deficient phenotype (data not shown). These microarray data, together with RNA gel blot analyses, indicate that *PEN3* is highly and ubiquitously expressed in plant tissues. These data further suggest that there is limited functional redundancy among the *PDR* genes and *PEN3*.

Publicly available expression data were mined to determine whether *PEN3* expression was modified after pathogen attack (see Supplemental Tables 2 and 3 online) (Zimmermann et al., 2004). Induction was modest after inoculation with virulent pathogens (e.g., *E. cichoracearum*, *Agrobacterium tumefaciens*, and the necrotroph *Plectosphaerella cucumerina*), inappropriate pathogens (e.g., *B. g. hordei*, *P. infestans*), and nonspecific elicitors (e.g., chitin fragments, flg22 peptide) but dramatic after inoculation with avirulent bacteria and the phloem-feeding aphid *Myzus persicae* (see Supplemental Table 2 online) (Glombitza et al., 2004; Zimmermann et al., 2004; De Vos et al., 2005; Ramonell et al., 2005). Induction by the flg22 peptide, a synthetic version of a conserved region of flagellin, was abolished in plants lacking FLS2, a receptor-like kinase required for flagellin perception (see Supplemental Table 2 online) (Gomez-Gomez and Boller, 2000). Exposure to methyl jasmonate, ozone, and the herbicides primisulfuron and prosulfuron also induced *PEN3* expression (see Supplemental Table 2 online) (Glombitza et al., 2004; Zimmermann et al., 2004). Although these two herbicides interfere with the synthesis of branched-chain amino acids, an herbicide that targets photosystem II, bromoxynil, did not induce *PEN3* expression (Glombitza et al., 2004). Abscisic acid and heat shock treatments downregulated the expression of *PEN3* (see Supplemental Table 2 online) (Busch et al., 2005). Thus, *PEN3* expression is altered by pathogens and a subset of abiotic stresses.

Resistance to *E. cichoracearum* and Cell Death in *pen3* Are SA-Dependent

To examine the possibility that *pen3-1* plants were also defective in basal resistance to virulent pathogens, they were infected with the *Arabidopsis* powdery mildew, *E. cichoracearum*. Surprisingly, *pen3-1* plants underwent chlorosis and cell death after *E. cichoracearum* infection (Figure 3A). Closer inspection indicated that cell death was fungus-associated and did not spread extensively beyond areas of fungal colonization (Figures 3C and 3E). By 5 d after inoculation (DAI), *E. cichoracearum* hyphae had elongated on wild-type leaves, leaving a trail of callose-containing papillae at each penetration site (Figure 3B). By contrast, hyphal elongation on *pen3-1* plants was accompanied by extensive callose deposition, preceding the death of penetrated cells (Figures 3C and 3E). By 7 DAI, extensive conidiation was observed on wild-type plants, whereas colonies that formed on *pen3-1* were stunted, lacked conidiophores, and were overlaid with patches of dead cells (Figure 3E).

Additionally, *pen3* plants were inoculated with the broad host range necrotrophs *Botrytis cinerea* and *P. cucumerina* (Berrocal-Lobo et al., 2002). *pen3-1* and *pen3-2* mutants were more susceptible to *P. cucumerina* than wild-type plants (Figure 4). Leaf tissue damage and fresh weight reduction caused by this fungus were greater in *pen3* mutants than in wild-type plants and similar to the damage observed in the highly susceptible *agb1-1* mutant, which is impaired in the β-subunit of *Arabidopsis* heterotrimeric G protein (Figure 4A) (Llorente et al., 2005). Resistance of *pen3-1* plants to *B. cinerea* did not differ from that of wild-type plants (data not shown).

Cell death in response to pathogen attack is often associated with the activation of the SA defense pathway (Greenberg and Yao, 2004). Similarly, chlorosis is associated with high levels of ET production (Schaller and Keiber, 2001). Double mutants were generated between *pen3-1* and mutants affecting the SA and ET/JA pathways to determine whether these defense signaling pathways were necessary for the pathogen-induced chlorosis and necrosis phenotype. Transgenic *NahG* plants, as well as mutants lacking a functional SA pathway, such as *eds1*, *pad4*, and *sid2*, suppressed the cell death and resistance phenotypes of *pen3-1*, allowing *E. cichoracearum* to conidiate (Figure 3F). The effect of

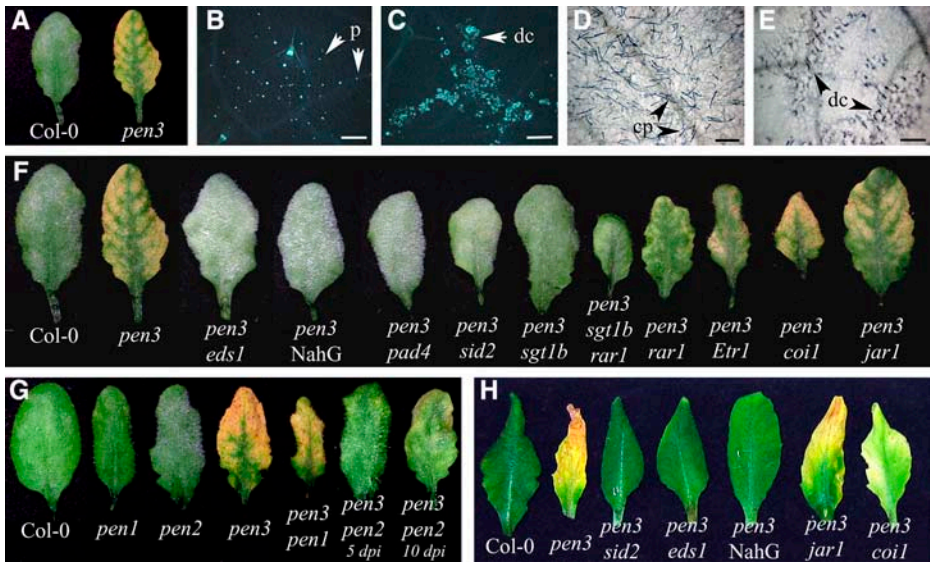


Figure 3. *pen3-1* Mutants Are Resistant to *E. cichoracearum* and Become Chlorotic after *E. cichoracearum* Infection.

(A) Single leaves at 7 DAI with the *Arabidopsis* powdery mildew, *E. cichoracearum*. Col-0 (left) supports good fungal conidiation, which is evident by eye as the whitish powdery appearance of inoculated leaves. Col-0 does not develop lesions, whereas *pen3-1*(right) undergoes chlorosis and cell death.
(B) and **(C)** On Col-0, *E. cichoracearum* infection elicits discrete callose deposits at papillae (p) **(B)**, whereas penetration attempts on *pen3-1* plants elicit widespread callose deposition and cell death (dc) **(C)**. Leaves were stained with aniline blue at 5 DAI. Bars = 100 μm.
(D) and **(E)** Lesions (dc) can be observed in *pen3-1* **(E)** but not in Col-0 **(D)** under conidiophores (cp) and hyphae. Leaves were stained with trypan blue at 7 DAI. Bars = 100 μm.
(F) Double mutant analysis to evaluate the role of the SA and ET/JA signal transduction pathways on resistance to *E. cichoracearum* in *pen3-1* plants. On double mutants with *pen3-1* and mutants or transgenic plants with blocks in the SA pathway (e.g., *eds1-1*, *NahG*, *pad4-1*, *sid2-1*), powdery mildew growth and conidiation exceed those observed in wild-type plants. The double mutant *pen3-1 sgt1b-1* and the triple mutant *pen3-1 sgt1b-1 rar1-10* support an intermediate level of powdery mildew conidiation that is slightly less than that observed on the wild type. Mutations in the ET pathway genes (e.g., *Etr1-1*), the JA pathway genes (e.g., *coi1-1*, *jar1-1*), and in *rar1-10* were not able to suppress the chlorotic and necrotic phenotype that develops on *pen3-1* mutants after infection with the *Arabidopsis* powdery mildew. Plants were photographed at 7 DAI.
(G) Double mutant analysis of the effect of *pen1-1* and *pen2-3* on *pen3-1*–associated resistance to *E. cichoracearum*. Plants were photographed at 6 DAI unless indicated otherwise.
(H) Double mutant analysis of the effect of the SA signal transduction pathway (*eds1-1*, *NahG*, *sid2-1*) and of the JA signal transduction pathway (*coi1-1*, *jar1-1*) on light-induced chlorosis of *pen3-1* plants. Plants were 4 weeks old when the photograph was taken.

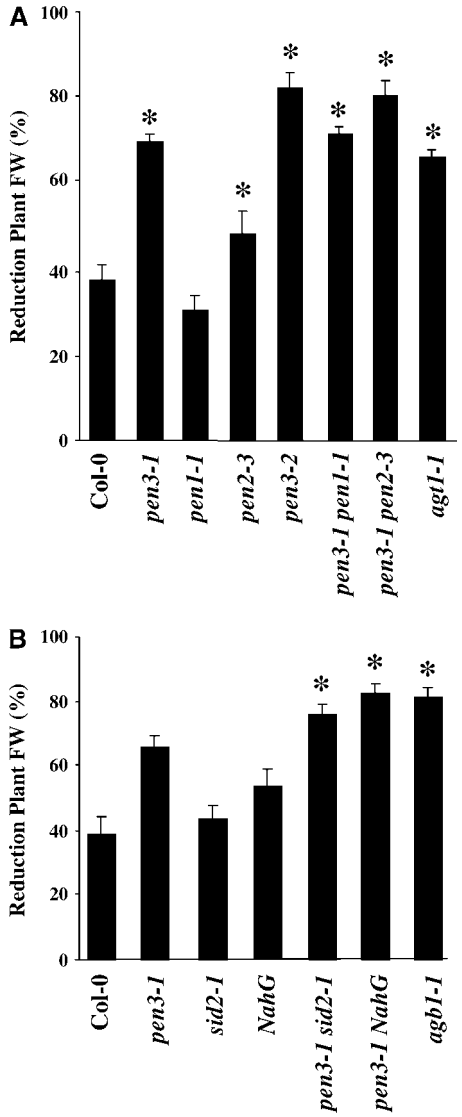


Figure 4. *pen3* Mutants Are More Susceptible to the Broad Host Range, Necrotrophic Pathogen *P. cucumerina*.

(A) *pen3* and *pen2* are highly susceptible to *P. cucumerina*. Plants were inoculated with a suspension of 2×10^6 spores/mL and scored at 7 DAI. * Significantly different from Col-0 using Student's *t* test ($P < 0.05$).

(B) The susceptibility to *P. cucumerina* of the *pen3-1* mutant does not depend on the SA signal transduction pathway. Plants were inoculated with a suspension of 1×10^6 spores/mL and scored at 10 DAI. * Significantly different from *pen3-1* using Student's *t* test ($P < 0.05$).

The susceptibility of plants was scored by fresh weight reduction (mean ± SD). At least 30 plants per genotype were inoculated with each pathogen, and the experiment was repeated four times. The highly susceptible *agb1-1* mutant was used as a positive control for disease development. FW, fresh weight.

two other SA-related mutants, *rar1* and *sgt1b*, on resistance to *E. cichoracearum* in *pen3-1* was also investigated. Mutations in *RAR1* did not affect the occurrence of cell death in inoculated *pen3-1* (Figure 3F). However, in *pen3-1 sgt1b* double mutants, conidiation was observed initially but chlorosis and cell death were delayed to ~10 DAI (data not shown). Triple *pen3-1 sgt1b rar1* mutants resembled *pen3-1 sgt1b* double mutants. Mutations that affect the ET/JA pathway, such as *jar1*, *coi1*, and *Etr1*, did not alter the *pen3-1* cell death phenotype. Thus, *pen3-1* resistance to the *Arabidopsis* powdery mildew is SA-dependent and ET/JA-independent.

Disease development after *P. cucumerina* infection was modestly enhanced in the SA-deficient lines *sid2-1* and *NahG*, as reported previously (Berrocal-Lobo et al., 2002), although to a lesser extent than in *pen3-1* (Figure 4B). The enhanced susceptibility to *P. cucumerina* observed in *pen3* mutants was further enhanced in *pen3-1 sid2-1* or *pen3-1 NahG* double mutant lines, suggesting that PEN3 acts additively with the SA pathway to limit *P. cucumerina* infections.

We considered the possibility that a propensity to become chlorotic and necrotic could indirectly result in increased pathogen entry. To address this question, a set of 34 *Arabidopsis* mutants that are resistant to the host powdery mildew pathogen were examined for *pen3*-like phenotypes (see Supplemental Table 4 online) (Greenberg and Ausubel, 1993; Frye and Innes, 1998; Rate et al., 1999; Clough et al., 2000; Petersen et al., 2000; Vogel and Somerville, 2000; Clarke et al., 2001; Mach et al., 2001; Rate and Greenberg, 2001; Maleck et al., 2002; Vogel et al., 2002, 2004; Liang et al., 2003). This collection included published *cpr*, *acd*, *agd*, and *pmr* mutants as well as a set of *mil* (for *mildew-induced lesion*) mutants (M. Nishimura, J. Vogel, and S. Somerville, unpublished data). Only one of these mutants, *mil9*, exhibited both increased penetration by *B. g. hordei* and resistance to *E. cichoracearum* (see Supplemental Table 4 online). The *PEN3* gene from *mil9* contained a point mutation at nucleotide 3985, which is predicted to change a Trp codon (TGG) to a stop codon (TGA). Thus, mutants prone to chlorosis and cell death do not commonly allow increased invasion by inappropriate pathogens.

Unlike *pen3*, *pen1* and *pen2* were not resistant to *E. cichoracearum* and did not become chlorotic upon infection (Collins et al., 2003; Lipka et al., 2005). As shown by the more rapid appearance of visible symptoms, *pen2* was more susceptible *to E. cichoracearum* than the wild type, indicating that PEN2 is important for both basal and nonhost resistance (Figure 3G). The *pen1* mutation was not able to suppress the *E. cichoracearum*–induced cell death of *pen3-1* (Figure 3G). In a *pen2-3 pen3-1* double mutant, however, cell death was delayed and resistance was compromised relative to the *pen3-1* mutant (Figure 3G). Although some conidiation occurred, *pen2-3 pen3-1* leaves underwent cell death and chlorosis at ~10 DAI (Figure 3G, data not shown). Thus, *pen3* resistance to *E. cichoracearum* plants is partially dependent on *PEN2*. In addition, *pen2*, but not *pen1*, was more susceptible to *P. cucumerina* than the wild type, but to a lesser degree than *pen3-1* and *pen3-2* (Figure 4A). The double mutants *pen3-1 pen1-1* and *pen3-1 pen2-3* resembled the *pen3* single mutants in their susceptibility to *P. cucumerina* (Figure 4A) (Lipka et al., 2005).

E. cichoracearum infection was not the only stress capable of inducing chlorosis and cell death in *pen3-1* plants. Although *pen3-1* plants were indistinguishable from wild-type plants up to 4 weeks after germination, eventually they became chlorotic and senesced earlier than wild-type plants (data not shown). This

phenotype was exacerbated by growth in continuous high light (24 h at 900 $\mu E \cdot m^{-2} \cdot s^{-1}$) (Figure 3H). High light–induced chlorosis and senescence were suppressed by mutations in the SA pathway but not by those in the ET/JA pathway (Figure 3H). Thus, the SA pathway appears to be necessary for heightened sensitivity to abiotic stress in *pen3-1* plants as well.

Effects of SA and ET/JA Pathway Mutations on Residual Nonhost Resistance in *pen3*

None of the mutations in the SA and ET/JA pathways tested reversed the loss of penetration resistance to *B. g. hordei* (Figure 5). However, plants carrying the *NahG* transgene and plants carrying mutations in *EDS1* were slightly compromised in their ability to restrict *B. g. hordei* entry into epidermal cells relative to wild-type plants (Figure 5) (Zimmerli et al., 2004). Microscopic inspection of the invasion and growth of *B. g. hordei* on double and single mutants revealed that the frequencies of fungal penetration on *pen3-1 NahG* and *pen3-1 eds1-1* were higher than those on the single mutants (Figure 5A). Colonies growing on these double mutant combinations were typically twice the average size than those growing on the single mutants (Figure 5B). This increase in growth was accompanied by an increase in hyphal branching (Figure 5C). Conidiation by *B. g. hordei* was not observed.

E. pisi epiphytic growth was increased on *eds1* and *pen3-1* mutants compared with wild-type plants (Figure 5D). A cumulative effect was seen on *pen3-1 eds1-1* double mutants, on which *E. pisi* colonies consisted of dense leaf-spanning mycelia, accompanied by occasional conidiophore formation (Figures 5D and 5E, insets). *E. pisi* conidia recovered from *Arabidopsis* double mutants were able to successfully infect pea and cause disease (data not shown).

Expression Profiling of *pen3-1* Plants

We compared the transcript profiles of wild-type and *pen3-1* plants at 1 DAI with *E. cichoracearum* or *B. g. hordei* using full-genome Affymetrix microarrays. Of the 22,810 probe sets (genes) on the microarray, 4240 exhibited some change in expression in this experiment. When considering the trends observed in the expression patterns of these genes, two main clusters were observed: genes induced by and genes repressed by pathogen attack (see Supplemental Figure 1 online). As described previously (Zimmerli et al., 2004), *B. g. hordei* elicited a more dramatic response than *E. cichoracearum*. Transcripts with increased levels after inoculation with the fungal pathogens included defense-related transcripts primarily, whereas transcripts with reduced levels consisted in large part of transcripts encoding photosynthetic and metabolic components (Zimmerli et al., 2004). It is possible that plants respond more dramatically to *B. g. hordei* because it cannot evade or suppress basal defenses as efficiently as the host powdery mildew, *E. cichoracearum*. In addition, responses to inoculation were more dramatic in *pen3-1* than in the wild type. Included among these genes were several SA-associated genes (Table 1). These included genes of the SA pathway, such as *PAD4*, *SID2*, *EDS1*, and *EDS5*, as well as downstream SA pathway markers, such as *PR-4*, *PR-5*, chitinases, and glucanases (Shah, 2003). This hyperactivation of SA defenses, together with the double mutant analysis, suggests that the basis for the resistance to *E. cichoracearum* observed in *pen3-1* plants is an enhanced activation of the SA pathway.

A single transcript (At3g30720) was highly upregulated in *pen3-1*–independent infection status ($P = 1.7 \times 10^{-8}$). This observation was confirmed by semiquantitative RT-PCR using independent samples (data not shown). In addition, the expression of this gene did not change very much upon inoculation with pathogens in either *pen3* or the wild type (i.e., Col-0 uninoculated, 78 intensity units [average of four replicates]; Col-0 + *E. cichoracearum*, 99; Col-0 + *B. g. hordei*, 74; *pen3-1* uninoculated, 1082; *pen3-1* + *E. cichoracearum*, 916; *pen3-1* + *B. g. hordei*, 978). No other gene exhibited this expression pattern. This gene is predicted to encode a 59–amino acid polypeptide with no significant homology with any known protein. ESTs have been found for this transcript, and array data show that this transcript responds to high CO_2 and is constitutively upregulated in the ET-insensitive *Etr1* mutant (Zimmermann et al., 2004). Unlike *pen3-1*, *Etr1* mutants resemble wild-type plants in their ability to restrict the entry of *B. g. hordei* into epidermal cells, and *Etr1* plants are susceptible to *E. cichoracearum* (data not shown). Thus, high levels of expression of this uncharacterized gene are not sufficient to account for the mutant phenotypes of *pen3-1*.

Of the *PDR* genes, only three, including *PEN3*, changed expression after inoculation with pathogens (see Supplemental Table 3 online) (Nishimura et al., 2003). Although *PDR4* was repressed after infection, *PDR12* was induced, and its induction was dramatically enhanced in *B. g. hordei*–inoculated *pen3-1* plants.

Localization of PEN3 to Penetration Sites after Pathogen Attack

In plants, ABC transporters have been localized to the tonoplast, mitochondria, chloroplasts, and plasma membrane (Kushnir et al., 2001; Moller et al., 2001; Goodman et al., 2004; Pighin et al., 2004). Recently, proteomic studies of different organelles have placed PEN3 in the plasma membrane, mitochondria, and chloroplasts (Brugiere et al., 2004; Kleffmann et al., 2004; Nühse et al., 2004). Given its high abundance, PEN3 is likely a common contaminant in organelle preparations and unlikely to be localized in all three subcellular structures. To localize PEN3, a translational fusion to green fluorescent protein (GFP) was made, transformed into *pen3-1* plants, and shown to complement the phenotypes conferred by the *pen3-1* mutant (data not shown). PEN3-GFP was localized to the plasma membrane; no GFP signal was associated with chloroplasts (Figure 6A). After inoculation with *B. g. hordei*, PEN3-GFP accumulated to high levels in regions of the plasma membrane just under fungal appressoria, in the shape of compact disks surrounded by diffuse halos approximately the size of papillae (Figures 6B to 6D). Occasionally, this disk of PEN3-GFP signal was surrounded by a concentric circle of intense PEN3-GFP label within the diffuse halo (Figure 6B). Closer inspection of PEN3-GFP fluorescence at attempted entry sites revealed a three-dimensional bubble-like shape, presumably reflecting the invagination of the plant plasma membrane by the growing fungal penetration peg and newly developing haustorium

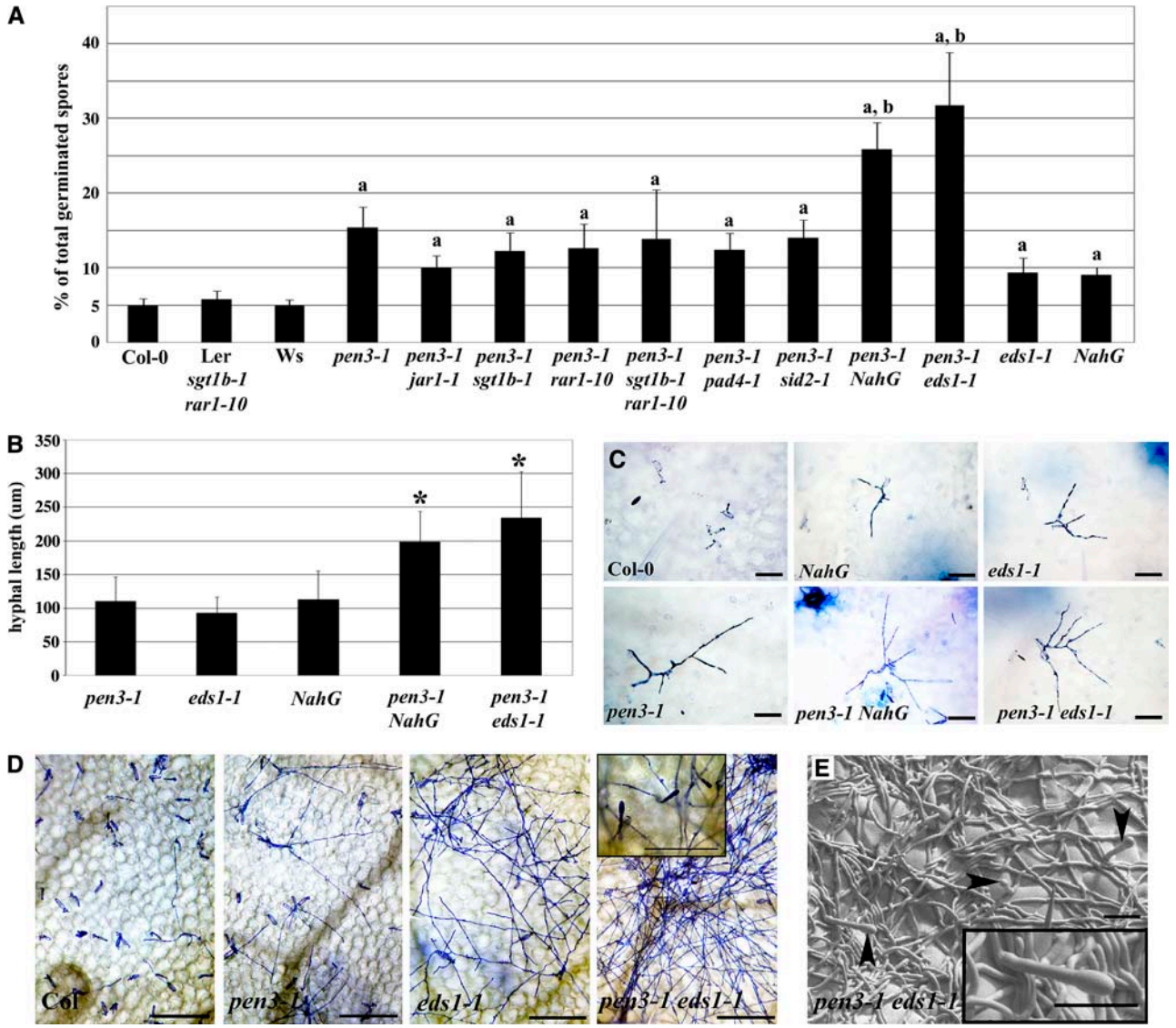


Figure 5. Combined Mutations in *PEN3* and *EDS1*or Introduction of the *NahG* Transgene Compromise Nonhost Resistance to *B. g. hordei* and *E. pisi*.

(A) Mean of the frequency of *B. g. hordei* penetration on *Arabidopsis*, expressed as a percentage of total germinated spores. Wassilewskija (Ws) is the wild-type control for *eds1-1*; L*er sgt1b-1 rar1-10* is the control for double mutants with *sgt1b-1* and *rar1-10*; and Col-0 is the wild-type control for *npr1-1*, *sid2-1*, *pad4-1*, *jar1-1*, and *NahG*. Eight leaves per genotype were stained with aniline blue at 2 DAI, and the occurrence of callose-encased haustoria was monitored as a measure of penetration efficiency. [a] Significantly different from the wild type; [b] significantly different from *pen3* using Student's *t* test ($P < 0.001$).

(B) Average hyphal length of *B. g. hordei* colonies growing on different mutants. Leaves were stained with trypan blue at 10 DAI, and hyphal lengths per colony were measured from photographs using ImageJ software. A minimum of 10 colonies were measured for hyphal length. This experiment was repeated twice. * Significantly different from the corresponding value for *pen3* using Student's *t* test ($P < 0.001$).

(C) Examples of *B. g. hordei* colonies growing on different lines. Infected leaves were stained with trypan blue at 10 DAI. Bars = 30 μm.

(D) *E. pisi* growth on Col-0 leaves and the indicated mutant genotypes at 7 DAI. Light microscopic images were taken after visualization of fungal structures using Coomassie Brilliant Blue. Bars = 200 μm. The inset shows a close-up view of *E. pisi* conidiophores on the *pen3-1 eds1-1*double mutant. Bar = 50 μm.

(E) Cryogenic scanning electron micrograph of *E. pisi* growth on *pen3-1 eds1-1* at 7 DAI. The inset shows an *E. pisi* conidiophore. Bars = 50 μm.

(Figures 6E and 6F). A funnel-like structure, which resembled a hollow tube and extended into the plant cell, was sometimes observed at penetration sites at later stages (Figures 6G and 6H). Localization of PEN3-GFP after *E. cichoracearum* infection was similar to that observed after inoculation with *B. g. hordei*. PEN3-GFP accumulated in compact disks beneath appressoria, as well as in diffuse halos around this domain, and in bubble-like structures (Figures 6I to 6K). When *E. cichoracearum* haustoria were formed, the PEN3-GFP marker was also observed partially surrounding the haustorium (Figure 6L).

Table 1. Examples of SA-Induced or SA Pathway–Associated Genes That Are Hyperinduced in *pen3-1* Mutants after Inoculation with Either the *Arabidopsis* or the Barley Powdery Mildew

Locus	Gene Description	Col-0 *Ec*	Col-0 *Bgh*	*pen3* Unin	*pen3* *Ec*	*pen3* *Bgh*
At1g65690	Harpin-induced protein–related	3.04	6.67	1.35	7.42	17.87
At1g74710	Isochorismate synthase1 (ICS1 = SID2)	2.23	5.67	1.15	4.13	10.14
At1g75040	Pathogenesis-related protein5 (PR-5)	4.89	9.18	2.55	12.81	26.13
At2g43570	Chitinase, putative similar to chitinase class IV	2.88	7.50	1.59	8.24	24.39
At2g43590	Chitinase, putative similar to basic endo chitinase CHB4	1.11	1.48	1.12	2.59	5.95
At2g46400	WRKY family transcription factor	2.16	4.80	1.52	3.63	8.42
At3g04720	Pathogenesis-related protein4 (PR4)	1.36	1.58	1.00	2.24	4.46
At3g12500	Basic endochitinase	1.05	1.28	1.14	2.37	8.39
At3g48090	Disease resistance protein, lipase-like (EDS1)	2.33	3.49	1.34	3.50	4.11
At3g50480	Broad-spectrum mildew resistance RPW8	2.61	4.97	1.52	5.35	11.19
At3g52430	Phytoalexin-deficient4 (PAD4)	3.20	5.48	1.42	5.02	9.75
At3g57240	β-1,3-Glucanase (BG3)	5.43	6.07	1.78	7.11	6.67
At4g01700	Chitinase, putative similar to peanut type II chitinase	3.79	6.91	1.36	6.28	8.26
At4g39030	Enhanced disease susceptibility5 (EDS5 = SID1)	2.44	5.43	1.13	4.84	10.33
At4g39830	L-Ascorbate oxidase	5.29	11.70	1.78	9.58	14.99
At5g24210	Lipase class 3 family protein	2.80	4.62	1.17	3.92	8.86
At5g47120	Bax inhibitor1 putative	1.57	2.23	1.15	2.13	3.19

Ec, 1 DAI with *Erysiphe cichoracearum*; Bgh, 1 DAI with *Blumeria graminis* f. sp *hordei*; Unin, uninoculated. Based on the analysis of variance with a Benjamini and Hochberg multiple text correction, the P values for these genes were <0.0008 for the factor genotype and for the factor infection. Values shown indicate average fold induction relative to uninoculated Col-0 ($n = 4$).

DISCUSSION

Like *pen1* and *pen2*, the *pen3* mutant allowed increased entry into epidermal cells and haustorium formation by the inappropriate pathogen *B. g. hordei* (Collins et al., 2003; Lipka et al., 2005). In addition, *pen3* supported increased hyphal elongation by the inappropriate fungus, indicating that a higher proportion of haustoria remained functional long enough to support the establishment and growth of secondary hyphae (Figure 1).

Nonhost resistance to *B. g. hordei* was also slightly compromised in the *eds1* mutant and in *NahG* transgenic plants, as shown for *B. g. tritici*, the powdery mildew pathogenic on wheat (Figure 5) (Yun et al., 2003; Zimmerli et al., 2004). Double mutants of *pen3-1* and *eds1* allowed additional penetration and growth by two separate inappropriate biotrophic pathogens, *B. g. hordei* and *E. pisi*, and the necrotroph *P. cucumerina*, than either mutant alone (Figures 4 and 5). Furthermore, *E. pisi* was able to reproduce asexually on plants with both *pen3-1* and *eds1-1* mutations (Figures 5D and 5E). Although EDS1 and NahG affect the functioning of the SA pathway, they both have additional poorly characterized but SA-independent effects on resistance (Feys et al., 2001; Heck et al., 2003; van Wees and Glazebrook, 2003). Because *sid2* and its double mutant with *pen3* did not implicate the SA pathway in nonhost resistance, it seems likely that the SA-independent responses of EDS1 and NahG contribute to nonhost resistance. Furthermore, double mutant combinations of *pen3* and *eds1* or *NahG* exhibited additive rather than epistatic interactions, suggesting that PEN3 operates independently of EDS1 and NahG in nonhost resistance. Therefore, it appears that only two barriers, penetration resistance (e.g., PEN2 or PEN3) and postpenetration or basal resistance (e.g., EDS1 or PAD4), are sufficient to limit *E. pisi* invasion, growth, and asexual reproduction on *Arabidopsis* (Lipka et al., 2005). However, an additional barrier(s) appears to limit *B. g. hordei* infections of *Arabidopsis*.

Unexpectedly, *pen3* mutants were more resistant to the *Arabidopsis* powdery mildew pathogen. This resistance was associated with the development of chlorosis and necrosis late in the infection sequence (Figure 3). A survey of 34 *Arabidopsis* mutants that exhibited chlorosis and necrosis upon pathogen infection showed that these phenotypes are not commonly associated with a lack of nonhost resistance (see Supplemental Table 4 online). Both double mutant analysis and microarray expression profiling suggested that the SA pathway was hyperactivated in *pen3* plants relative to wild-type plants and that this was responsible for the chlorotic and *E. cichoracearum*–resistant phenotypes of *pen3* (Table 1, Figure 3). SGT1b has a role in cell death signaling in addition to its role in modulating R protein levels, which is consistent with the attenuation of chlorosis and cell death observed in *pen3 sgt1b* double mutants (Figure 3) (Holt et al., 2005). Because powdery mildews are obligate biotrophs that grow only on living host tissue, the necrosis and chlorosis are likely responsible for the resistance to *E. cichoracearum* in *pen3* plants.

PEN3 encodes a putative PDR-like ABC transporter previously designated *PDR8*. The *Arabidopsis PDR* gene family comprises 15 members, of which *PEN3* is the most widely and highly

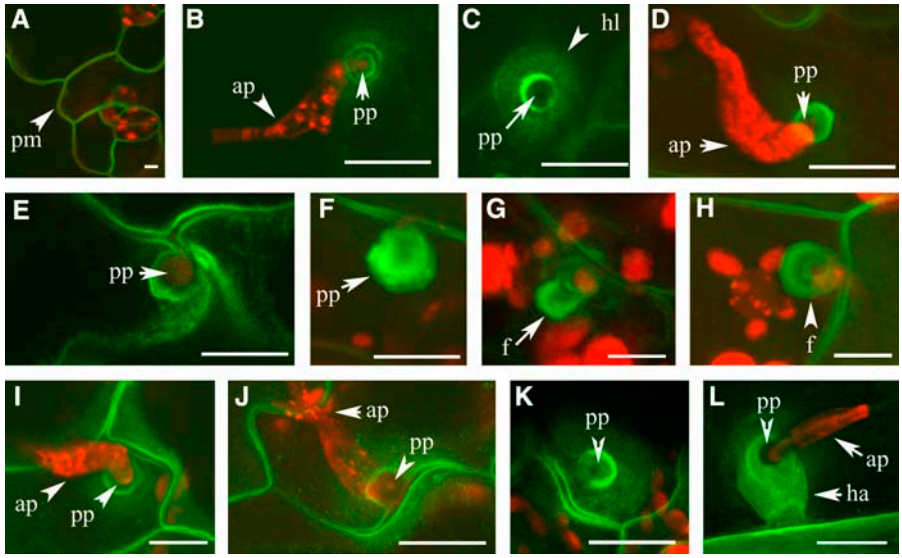


Figure 6. Localization of PEN3-GFP.

(A) PEN3-GFP localizes to plant plasma membranes (pm) in uninfected leaves.
(B) to **(H)** Localization of PEN3-GFP in leaves inoculated with *B. g. hordei*.
(B) Conidia germinate and produce appressoria (ap), which attempt penetration via penetration pegs (pp).
(C) Green channel image of an attempted penetration site, accompanied by the accumulation of PEN3-GFP directly around the peg and by diffuse accumulation in a halo-like structure (hl).
(D) PEN3-GFP accumulates preferentially beneath penetration pegs.
(E) and **(F)** Accumulation of PEN3-GFP around the penetration peg persists in a bubble-like structure as penetration continues.
(G) and **(H)** At later stages, PEN3-GFP is seen in a funnel-like structure (f) around haustorial initials. This structure extends deep into the cell (side view **[G]**) and does not fully encase the haustorial initial (bottom view **[H]**).
(I) to **(L)** Localization of PEN3-GFP in leaves inoculated with *E. cichoracearum*.
(I) to **(K)** Attempted penetrations by *E. cichoracearum* elicit PEN3-GFP aggregation around penetration pegs and in diffuse halos.
(L) At later stages, PEN3-GFP aggregates around the upper part of the haustoria (ha).
PEN3-GFP appears green; chloroplast autofluorescence and propidium iodide–stained fungal structures appear red. Bars = 5 μm.

expressed. Although other ABC transporter families in plants are well characterized and their members have been implicated in the transport of auxin, lipids, pigments, and chlorophyll precursors, the PDR family is less well studied (Martinoia et al., 2002). Among the plant PDR subgroup of ABC transporters, studies of two *Nicotiana* species transporters, *N. plumbaginifolia* PDR1/ABC1 and tobacco (*Nicotiana tabacum*) PDR1, which are most similar in sequence to the *Arabidopsis* PDR12, suggest that they may function in the export of toxic secondary plant metabolites and/or in the detoxification of pathogen toxins. Similar to *PEN3*, the expression of *N. tabacum PDR1* and *N. plumbaginifolia PDR1* is induced by defense-related signals such as pathogen elicitors and methyl jasmonate, and *N. plumbaginifolia PDR1* is also induced by the phytoalexin sclareol (Jasinski et al., 2001; Sasabe et al., 2002). *Arabidopsis* does not produce sclareol; however, *PDR12* is induced by fungal pathogens, and plants carrying a T-DNA insertion in *PDR12* are reportedly more sensitive to exogenous sclareol and to lead (see Supplemental Table 3 online) (Campbell et al., 2003; Lee et al., 2005). Given the broad range of compounds and small peptides that can be transported by ABC transporters, these studies of tobacco PDR transporters cannot be used to suggest a candidate substrate for PEN3. A single gene, encoding a small unknown protein, was strongly upregulated in the *pen3* mutant independent of infection status. In yeast, an ABC transporter is responsible for exporting the 38–amino acid mating factor A (Kuchler et al., 1989). Thus, it is possible that the small unknown protein is a substrate for PEN3. Alternatively, this gene could be a novel marker for the stress experienced by *pen3* plants. In spite of the expression responses, loss of PDR12 function in *Arabidopsis* or PDR1 function in *N. plumbaginifolia* did not lead to increased susceptibility to several fungal and bacterial pathogens (Campbell et al., 2003; Stukkens et al., 2005). However, *N. plumbaginifolia* plants lacking PDR1 were highly susceptible to the necrotroph *B. cinerea* (Stukkens et al., 2005). With this result and the cloning of *PEN3*, PDR-type ABC transporters have been implicated in defense, adding a new role to the diverse repertoire of the PDR-like ABC transporter family.

ABC transporters have been shown to require phosphorylation for activity or regulation (Kolling, 2002). A recent study of phosphorylated plasma membrane proteins identified PEN3 as a target of flagellin-induced phosphorylation (Nühse et al., 2004), supporting the idea that PEN3 functions in nonhost resistance and defenses elicited by nonspecific elicitors such as flagellin. Consistent with this idea, *PEN3* expression was 2.5-fold higher in flg22 peptide–treated seedlings compared with control seedlings, and

this induction was dependent on the FLS2 receptor-like kinase (see Supplemental Table 2 online) (Gomez-Gomez and Boller, 2000).

Localization of PEN3 to the plasma membrane and its accumulation at penetration sites support the idea that PEN3 exports defense compounds in a focal manner at attempted invasion sites (Figure 6). PEN1 (=SYP121 syntaxin), the barley PEN1 homolog ROR2, and the barley MLO protein also localize in a similar pattern to the plasma membrane after powdery mildew infection (Assaad et al., 2004; Bhat et al., 2005). In addition, PEN2-GFP localizes to peroxisomes that accumulate near sites of invasion (Lipka et al., 2005). This marshalling of defense components at sites of attempted pathogen invasion illustrates the importance of subcellular dynamics in plant responses to pathogens.

pen3 and *pen2* mutants have similar phenotypes that are not observed in *pen1* mutants. For example, only *pen2* and *pen3* plants were more susceptible to the inappropriate pathogen *P. infestans* (Figures 1G and 1I) and the necrotroph *P. cucumerina* (Figure 4) (Lipka et al., 2005). However, *pen3* mutants differed from *pen2* mutants by their resistance to the *Arabidopsis* powdery mildew (Figure 3A). Mutations in *PEN2* partially suppressed cell death and chlorosis in *E. cichoracearum*–infected *pen3* mutants, suggesting that PEN2 and PEN3 functions might be interdependent. We propose a biochemical model for the role of PEN3 in penetration resistance (Figure 7A) in which PEN2 converts a nontoxic substrate to a toxic product, which is then exported either directly or after further modification to the apoplast by PEN3, poisoning the fungal penetration peg as it attempts to cross the cell wall. This model predicts that the substrate(s) normally exported by PEN3 accumulates to higher levels intracellularly in *E. cichoracearum*–infected *pen3* plants than in *pen3* plants inoculated with *B. g. hordei*, because *E. cichoracearum* continues to grow and repeatedly penetrate the epidermis, providing a greater stimulus for the production of PEN3 substrate(s) (Figure 7B). Based on the double mutant analysis (Figure 3) and microarray data (Table 1), it seems likely that the PEN3 substrate(s) spuriously activates the SA pathway. Because *pen3* but not *pen2* mutants develop chlorotic and necrotic patches with *E. cichoracearum* infections, we further propose that another enzyme(s), acting in parallel with PEN2, generates a related toxin(s) that is also exported by PEN3. To date, one phytoalexin, camalexin, has been identified in *Arabidopsis* (Tsuji et al., 1992). However, it is unlikely that this compound is solely responsible for the phenotypes conferred by *pen3*, because *phytoalexin-deficient3* (*pad3*) and *pad4* mutants lack the phenotype conferred by *pen* (data not shown). A postpenetration role for PEN3 can be explained by suggesting that PEN3 exports toxin(s) to the extrahaustorial matrix, poisoning the haustorium, thereby limiting the initiation and growth of secondary hyphae. This suggestion is supported by the localization of PEN3 protein to haustorial complexes (Figure 6). High-light stress presumably leads to the accumulation of toxic materials that would normally be exported by *PEN3*. The data supporting this model are incomplete as yet, and it is also possible that the loss of *PEN2* attenuates the activation of the SA pathway in *pen3* mutants or that PEN3 exports a fungal toxin or a fungal suppressor of defenses.

Plants are resistant to most pathogens in their environment and succumb to only a small number of highly adapted pathogen species. Collectively, the isolation of the *pen1*, *pen2*, and *pen3* mutants highlights the importance of defenses operating at the cell periphery. Only a subset of pathogens are able to overcome

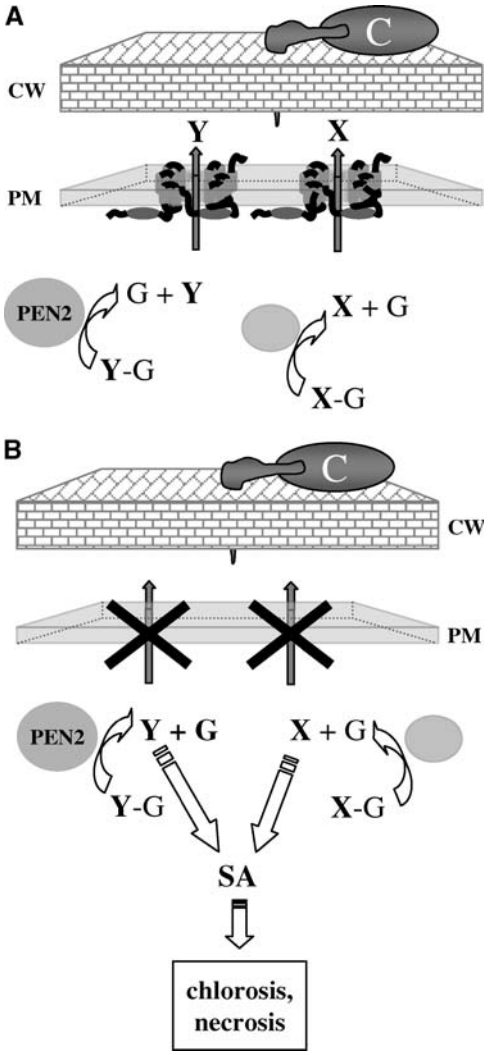


Figure 7. Model for a Role for PEN3 in Penetration Resistance.

(A) We hypothesize that the PEN3 ABC transporter exports toxic secondary metabolites or other toxic materials (X and Y) to the apoplast at sites of attempted invasion. We speculate that the barley and pea powdery mildews are sensitive to these toxins, whereas the *Arabidopsis* powdery mildew is either less sensitive to this toxin or fails to fully activate this defense mechanism. Because some ABC transporters can transport a variety of related compounds, we assume that PEN3 exports a family of chemically related compounds, which may have varying toxicity. The glycosyl hydrolase PEN2 may activate one of these toxins (Y) by hydrolysis of a nontoxic precursor metabolite (Y-G). We assume that other enzymes activate related toxins (X).
(B) In the *pen3* mutant, the sustained intracellular accumulation of PEN2-activated and related toxin(s) in interactions with the *Arabidopsis* powdery mildew leads to the activation of the SA pathway and the development of chlorosis and necrosis.
C, conidium; CW, cell wall; PM, plasma membrane.

these defenses and attempt to colonize plants when additional defenses, operating intracellularly, come into play. For some inappropriate pathogens, only a limited number of barriers limit growth and asexual reproduction, suggesting that nonhost resistance may not be as complex as originally thought and that these defenses from nonhost plants can be introduced into host plants to provide stable, broad host range resistance to difficult pathogens.

METHODS

Growth Conditions and Inoculations

Arabidopsis thaliana and squash (*Cucurbita maxima*, Hybrid Kuta; Park Seed, Greenwood, SC) were grown in growth chambers at 22°C with a 14-h photoperiod of ~125 $\mu E \cdot m^{-2} \cdot s^{-1}$ in the 400- to 700-nm range. Host powdery mildew (*Erysiphe cichoracearum* UCSC1) was cultured on squash for 10 to 12 d and then applied to *Arabidopsis* using settling towers (Vogel and Somerville, 2000). Barley powdery mildew (*Blumeria graminis* f. sp *hordei* CR3) was grown on barley (*Hordeum vulgare*) line Algerian-S (CI-16138) and inoculated onto *Arabidopsis* using the methods described by Zimmerli et al. (2004). *Phytophthora infestans* isolate 1306 (A1 mating type; provided by H. Judelson, University of California, Riverside) was maintained and prepared for inoculations as described by Zimmerli et al. (2004). Plants to be infected with *Erysiphe pisi* were grown in growth chambers at 20 to 23°C with a 12-h photoperiod and a light intensity of ~150 $\mu E \cdot m^{-2} \cdot s^{-1}$ on a turf substrate mix (Stender Substrate; Wesel-Schermbeck) containing 0.001% Confidor WG70 (Bayer). Three-week-old plants were inoculated with *E. pisi* (Birmingham Isolate) using a settling tower (Lipka et al., 2005). For the growth of *Plectosphaerella cucumerina* and *Botrytis cinerea*, seeds were surface-sterilized, sown on square, 12.5 × 12.5-cm Petri dishes containing Murashige and Skoog basal salt mixture medium with 0.8% phytagel (Sigma-Aldrich), transferred to a phytochamber, and grown as described previously (Berrocal-Lobo et al., 2002; Berrocal-Lobo and Molina, 2004; Llorente et al., 2005). For high-light treatments, plants were germinated and grown at 22°C under a continuous light regime of ~900 $\mu E \cdot m^{-2} \cdot s^{-1}$ in the 400- to 700-nm range.

M2 seeds were derived from an ethyl methanesulfonate–mutagenized population of Col-0 and screened for loss of penetration resistance to *B. g. hordei* by identifying individuals supporting an increased frequency of haustorium formation. The *B. g. hordei* haustoria became encased in callose and were visualized by staining for callose as described below

Cytology and Quantification of Fungal Growth

For penetration assays, 3-week-old *Arabidopsis* plants were inoculated with *B. g. hordei* and leaf samples were taken at 2 DAI. For *P. infestans* assays, 2-week-old *Arabidopsis* plants were sprayed with *P. infestans* zoospore suspensions and leaves were sampled at 2 DAI (Zimmerli et al., 2000). The deposition of callose was visualized by staining with aniline blue (Vogel and Somerville, 2000). For *E. cichoracearum* infections, 3-week-old *Arabidopsis* plants were inoculated as described above, and phenotypes were monitored visually at 4, 5, 6, 7, and 10 DAI.

To quantify fungal growth, eight *Arabidopsis* leaves per genotype were stained with aniline blue as described by Adam and Somerville (1996), with 250 mg of trypan blue added per milliliter. Haustoria, which become encased in callose in incompatible interactions, were visualized with aniline blue staining. Secondary hyphal elongation was quantified in nonoverlapping fields of view at ×200 magnification, avoiding the edge and midvein regions of the leaf. Numbers were expressed as percentages of total germinated conidia and were assessed for differences between mutant and the wild type with Student's *t* test. Fungal growth, the occurrence of lesions, and the accumulation of autofluorescent compounds were assessed as described by Vogel and Somerville (2000). To quantify hyphal length per colony, *B. g. hordei*–inoculated leaves were harvested at 10 DAI, cleared, and stained with trypan blue (Vogel and Somerville, 2000). At least 10 individual colonies were photographed under bright-field illumination at ×200 magnification with a Leica D500 digital camera attached to a Leica Leitz DMRB compound microscope. Hyphal lengths were measured using ImageJ software (version 1.30) (Abramoff et al., 2004; http://rsb.info.nih.gov/ij/).

Leaves infected with *E. pisi* were fixed and cleared in ethanol:acetic acid (3:1). Epiphytic fungal growth was visualized by staining of fungal structures with an ethanolic solution containing 0.6% Coomassie Brilliant Blue R 250 (Lipka et al., 2005). Cryogenic scanning electron microscopy of *E. pisi*–infected plants was performed as described by Sturaro et al. (2005).

Assays of *P. cucumerina* disease development were done by spraying 10-d-old plants with a spore suspension (at either 1×10^6 or 2×10^6 spores/mL, 1.5 mL/plate). Mock inoculations were done by spraying the plants with sterile water. After inoculation, plants were kept under the growth conditions described above; 10 d later, the percentage reduction of plant fresh weight caused by the fungal infection was calculated as described (Berrocal-Lobo and Molina, 2004). Inoculations of wild-type plants and *pen3-1* mutants with *B. cinerea* were performed as described previously (Llorente et al., 2005), and the percentage of decayed plants was determined at different days after inoculation. The highly susceptible *agb1-1* mutant was included in these experiments as a control for disease development (Llorente et al., 2005). At least 30 plants per genotype were inoculated with each pathogen, and the experiment was repeated four times. Differences between genotypes were identified using Student's *t* test.

Mapping and Cloning

Rough mapping markers, PCR conditions, and DNA preparation were as described previously (Lukowitz et al., 2000). Briefly, F2 populations of a mutant crossed to *Arabidopsis* L*er* were sown in 96-well flats and then moved to a greenhouse. Plants were inoculated with *B. g. hordei*, as described above, at ~3 weeks after germination when they had at least four true leaves. At 2 DAI, the third or fourth true leaf from each plant was harvested. Leaves were stained individually, mounted, and examined at ×50 magnification as described below. Tissue was harvested from plants with mutant phenotypes for DNA preparation and bulk segregant PCR (Lukowitz et al., 2000). Key individuals were retested for their *pen3* phenotype in the F3 generation. New markers within the mapping interval were generated using the CEREON database of Col-0 and L*er* polymorphisms (Jander et al., 2002) and the Whitehead Institute online primer design software, Primer3 (Rozen and Skaletsky, 2000; http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). All available T-DNA insertion lines for genes in a small interval on chromosome 1 were identified from the Salk Institute Genomic Analysis Laboratory's T-DNA project (http://signal.salk.edu/cgi-bin/tdnaexpress) (Ecker, 2002). Two SALK lines, SALK_110926 (*pen3-3*) and SALK_000578 (*pen3-4*), with insertions in the At1g59870 gene segregated *pen3* phenotypes. The At1g59870 gene was sequenced from the *pen3-1*, *pen3-2*, and *mil9* mutant lines on an ABI 310 Sequenator (Applied Biosystems).

Alignments of *PDR* family members were made using DNAStar MEGALIGN software with the ClustalW method and default parameters. Microarray data mining was done using the web tool GENEVESTIGATOR (Zimmermann et al., 2004).

Double Mutant Construction

The mutant alleles used for the construction of double mutants with *pen3-1* were *coi1-1* (Col-6 background [Feys et al., 1994]), *npr1-1* (Cao et al.,

1994), *sid2-1* (Nawrath and Métraux, 1999), *pad4-1* (Glazebrook and Ausubel, 1994), *eds1-1* (Ws-0 background [Parker et al., 1996]), *Etr1-1* (Bleecker et al., 1988), *jar1-1* (Staswick et al., 1992), *edr1* (Frye and Innes, 1998), *sgt1b-1* (L*er* background [Peart et al., 2002]), *rar1-10* (L*er* background [Muskett et al., 2002]), *NahG* (Lawton et al., 1995), *pen1-1* (Collins et al., 2003), and *pen2-3* (Lipka et al., 2005). All of the mutant lines, unless noted otherwise, were in the Col-0 background. We generated the double mutants by standard genetic crosses, following the mutations with cleaved-amplified polymorphic sequence (CAPS) markers. When the mutation did not lead to a change in a restriction site, we generated derived CAPS markers using the dCAPS web tool (Neff et al., 1998). The *Etr1-1* (Hua and Meyerowitz, 1998), *NahG* (Morris et al., 2000), *rar1-10* (Muskett et al., 2002), *sgt1b* (Austin et al., 2002), *coi1-1* (Xie et al., 1998), *pad4-1* (Nishimura et al., 2003), and *npr1-1* (Nishimura et al., 2003) primers have been described previously. See Supplemental Table 5 online for a listing of primers used in genotyping mutations.

Expression Profiling

Samples of Col-0 and *pen3-1* rosettes were collected from uninfected plants, plants infected with *E. cichoracearum*, or plants inoculated with *B. g. hordei* at 1 DAI. Each sample represented a pool of the rosettes from 16 plants grown in one pot under the growth conditions outlined above and inoculated as described above. Four pots (each pot a biological replicate) were grown for each treatment × genotype combination for a total of 24 pots. Total RNA was extracted from the plants using the Trizol method (Chomczynski and Sacchi, 1987) (Gibco BRL) and purified (Qiagen; RNeasy) essentially as described (Ramonell et al., 2002). Biotinylated complementary RNA (20 μg) was prepared as described (Hernan et al., 2003). The resulting complementary RNA was used to hybridize ATH1 Arabidopsis GeneChips (Affymetrix) using the manufacturer's protocols. The array images were analyzed with Affymetrix Microarray Suite 5.0 with the target intensity set to 500. The expression levels of the genes were analyzed with GeneSpring 4.2 software (Silicon Genetics), and the chip-to-chip signal variation was minimized by normalizing signal intensities to the averaged intensity values of uninoculated Col-0 using the expression levels of the top 50th percentile of probe sets. Differentially expressed genes were identified using two-way analysis of variance and a Benjamini and Hochberg multiple testing correction (GeneSpring 4.2). Genes were considered differentially expressed at $P \leq 0.001$.

PEN3-GFP Localization

To create a PEN3 fusion to GFP, a 3′ fragment encoding the C terminus (nucleotides 4380 to 5681 of the unspliced transcript) of *PEN3* was amplified by PCR using primers containing a C-terminal adaptor to keep the protein sequence in-frame with GFP in the vector pEGAD (Cutler et al., 2000). The primer sequences were 5′-CGGGGTACCGGACACTGGAA-GAACCGTGGTC-3′ and 5′-ACGCGTCGACCGTCCCAACATGGAAACT-CTTGTATC-3′. The 35S promoter was removed from pEGAD using *Sac*I, before ligating the PCR-amplified and *Kpn*I-*Bam*HI–digested C-terminal *PEN3* fragment in-frame with eGFP. BAC F23H11 was ordered from the ABRC and digested with *Kpn*I, yielding a 10-kb genomic fragment containing the *PEN3* gene as well as 2132 nucleotides upstream of the start codon and 2542 nucleotides downstream of the stop codon. This fragment was gel-purified and redigested using *Dra*III. The resulting digest was cloned into the pEGAD vector containing the *PEN3* sequences encoding the C terminus fused with *eGFP* and digested with *Kpn*I and *Dra*III, to give the full genomic piece fused in-frame to GFP. The vector was transformed into *Agrobacterium tumefaciens* (strain GV3101), and flowering *pen3-1* plants were dipped into *A. tumefaciens*–containing infiltration medium as described (Cutler et al., 2000). T1 seeds were surface-disinfected and plated on Murashige and Skoog agar plates containing 50 μg kanamycin/mL (Murashige and Skoog, 1962). Two-week-old healthy seedlings were screened for GFP fluorescence with a Leica dissecting microscope equipped with an epifluorescence filter set. GFP-expressing seedlings were transferred to soil and inoculated with *E. cichoracearum* or *B. g. hordei* 2 weeks later. Single inoculated leaves from T1 plants were mounted on microscope cover slips in a 0.01 mg/mL propidium iodide solution in water (Ramonell et al., 2005). Imaging was done using a Nikon inverted fluorescence microscope equipped with a Bio-Rad MRC 1024 confocal head. Images were processed using the software ImageJ.

Accession Numbers

Microarray data sets were deposited in the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/; Barrett et al., 2005) under accession number GSE3220. The Arabidopsis Genome Initiative locus identifier for *PEN3*/*PDR8* is At1g59870.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Table 1. Expression of the 12 Members of the *Arabidopsis PDR* Family in Various Tissues.

Supplemental Table 2. *PEN3* Transcript Levels Are Increased under Different Biotic and Abiotic Stresses.

Supplemental Table 3. Changes in Transcript Levels of *PDR* Family Members upon Powdery Mildew Infection in Wild-Type and *pen3-1* Plants.

Supplemental Table 4. Host and Nonhost Resistance Phenotypes of Mutants Resistant to *E. cichoracearum*.

Supplemental Table 5. Primer Combinations Used to Genotype Mutations by PCR.

Supplemental Figure 1. Transcript Profiling of *pen3-1* and Col-0 after Inoculation with the *Arabidopsis* and Barley Powdery Mildew Pathogens.

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REFERENCES

Abramoff, M.D., Magelhaes, P.J., and Ram, S.J. (2004). Image processing with ImageJ. Biophotonics International **11,** 36–42.

Adam, L., and Somerville, S.C. (1996). Genetic characterization of five powdery mildew disease resistance loci in *Arabidopsis thaliana.* Plant J. **9,** 341–356.

Assaad, F.F., Qiu, J.L., Youngs, H., Ehrhardt, D., Zimmerli, L., Kalde, M., Wanner, G., Peck, S.C., Edwards, H., Ramonell, K., Somerville, C.R., and Thordal-Christensen, H. (2004). The PEN1 syntaxin defines a novel cellular compartment upon fungal attack and is required for the timely assembly of papillae. Mol. Biol. Cell **15,** 5118–5129.

Austin, M.J., Muskett, P., Kahn, K., Feys, B.J., Jones, J.D.G., and Parker, J.E. (2002). Regulatory role of SGT1 in early *R* gene-mediated plant defenses. Science **295,** 2077–2080.

Barrett, T., Suzek, T.O., Troup, D.B., Wilhite, S.E., Ngau, W.C., Ledoux, P., Rudnev, D., Lash, A.E., Fujibuchi, W., and Edgar, R. (2005). NCBI GEO: Mining millions of expression profiles—Database and tools. Nucleic Acids Res. **33,** D562–D566.

Berrocal-Lobo, M., and Molina, A. (2004). *ETHYLENE RESPONSE FACTOR 1* mediates Arabidopsis resistance to the soilborne fungus *Fusarium oxysporum.* Mol. Plant Microbe Interact. **17,** 763–770.

Berrocal-Lobo, M., Molina, A., and Solano, R. (2002). Constitutive expression of *ETHYLENE-RESPONSE-FACTOR1* in Arabidopsis confers resistance to several necrotrophic fungi. Plant J. **29,** 23–32.

Bhat, R.A., Miklis, M., Schmelzer, E., Schulze-Lefert, P., and Panstruga, R. (2005). Recruitment and interaction dynamics of plant penetration resistance components in a plasma membrane microdomain. Proc. Natl. Acad. Sci. USA **102,** 3135–3140.

Bleecker, A.B., Estelle, M., Somerville, C., and Kende, H. (1988). Insensitivity to ethylene conferred by a dominant mutation in *Arabidopsis thaliana.* Science **241,** 1086–1089.

Braun, U., Cook, R.T.A., Inman, A.J., and Shin, H.-D. (2002). The taxonomy of the powdery mildew fungi. In The Powdery Mildews, A Comprehensive Treatise, R.R. Belanger, W.R. Bushnell, A.J. Dik, and T.L.W. Carver, eds (St. Paul, MN: APS Press), pp. 13–55.

Brugiere, S., Kowalski, S., Ferro, M., Seigneurin-Berny, D., Miras, S., Salvi, D., Ravanel, S., d'Herin, P., Garin, J., Bourguignon, J., Joyard, J., and Rolland, N. (2004). The hydrophobic proteome of mitochondrial membranes from Arabidopsis cell suspensions. Phytochemistry **65,** 1693–1707.

Busch, W., Wunderlich, M., and Schoffl, F. (2005). Identification of novel heat shock factor-dependent genes and biochemical pathways in *Arabidopsis thaliana.* Plant J. **41,** 1–14.

Campbell, E.J., Schenk, P.M., Kazan, K., Penninckx, I.A.M.A., Anderson, J.P., Maclean, D.J., Cammue, B.P.A., Ebert, P.R., and Manners, J.M. (2003). Pathogen-responsive expression of a putative ATP-binding cassette transporter gene conferring resistance to the diterpenoid sclareol is regulated by multiple defense signaling pathways in Arabidopsis. Plant Physiol. **133,** 1272–1284.

Cao, H., Bowling, S.A., Gordon, A.S., and Dong, X.N. (1994). Characterization of an *Arabidopsis* mutant that is nonresponsive to inducers of systemic acquired resistance. Plant Cell **6,** 1583–1592.

Chomczynski, P., and Sacchi, N. (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. **162,** 156–159.

Clarke, J.D., Aarts, N., Feys, B.J., Dong, X.N., and Parker, J.E. (2001). Constitutive disease resistance requires EDS1 in the Arabidopsis mutants *cpr1* and *cpr6* and is partially EDS1-dependent in *cpr5.* Plant J. **26,** 409–420.

Clough, S.J., Fengler, K.A., Yu, I.C., Lippok, B., Smith, R.K., and Bent, A.F. (2000). The Arabidopsis *DND1* ''defense, no death'' gene encodes a mutated cyclic nucleotide-gated ion channel. Proc. Natl. Acad. Sci. USA **97,** 9323–9328.

Collins, N.C., Thordal-Christensen, H., Lipka, V., Bau, S., Kombrink, E., Qiu, J.L., Hückelhoven, R., Stein, M., Freialdenhoven, A., Somerville, S.C., and Schulze-Lefert, P. (2003). SNARE-protein-mediated disease resistance at the plant cell wall. Nature **425,** 973–977.

Cutler, S.R., Ehrhardt, D.W., Griffitts, J.S., and Somerville, C.R. (2000). Random GFP:cDNA fusions enable visualization of subcellular structures in cells of Arabidopsis at a high frequency. Proc. Natl. Acad. Sci. USA **97,** 3718–3723.

De Vos, M., Van Oosten, V.R., Van Poecke, R.M.P., Van Pelt, J.A., Pozo, M.J., Mueller, M.J., Buchala, A.J., Métraux, J.P., Van Loon, L.C., Dicke, M., and Pieterse, C.M.J. (2005). Signal signature and transcriptome changes of Arabidopsis during pathogen and insect attack. Mol. Plant Microbe Interact. **18,** 923–937.

Ecker, J.R. (2002). A sequence-indexed library of insertion mutations in the Arabidopsis genome. Plant Physiol. **129,** 405–406.

Ellingboe, A.H. (1972). Genetics and physiology of primary infection by *Erysiphe graminis.* Phytopathology **62,** 401–406.

Fernandez, M.R., and Heath, M.C. (1991). Interactions of the nonhost French bean plant *Phaseolus vulgaris* with parasitic and saprophytic fungi. 4. Effect of preinoculation with the bean rust fungus on growth of parasitic fungi nonpathogenic on beans. Can. J. Bot. **69,** 1642–1646.

Feys, B.J., Benedetti, C., Penfold, C., and Turner, J.G. (1994). *Arabidopsis* mutants selected for resistance to the phytotoxin coronatine are male sterile, insensitive to methyl jasmonate, and resistant to a bacterial pathogen. Plant Cell **6,** 751–759.

Feys, B.J., Moisan, L.J., Newman, M.A., and Parker, J.E. (2001). Direct interaction between the Arabidopsis disease resistance signaling proteins EDS1 and PAD4. EMBO J. **20,** 5400–5411.

Frye, C.A., and Innes, R.W. (1998). An *Arabidopsis* mutant with enhanced resistance to powdery mildew. Plant Cell **10,** 947–956.

Glazebrook, J., and Ausubel, F.M. (1994). Isolation of phytoalexin-deficient mutants of *Arabidopsis thaliana* and characterization of their interactions with bacterial pathogens. Proc. Natl. Acad. Sci. USA **91,** 8955–8959.

Glombitza, S., et al. (2004). Crosstalk and differential response to abiotic and biotic stressors reflected at the transcriptional level of effector genes from secondary metabolism. Plant Mol. Biol. **54,** 817–835.

Gomez-Gomez, L., and Boller, T. (2000). FLS2: An LRR receptor-like kinase involved in the perception of the bacterial elicitor flagellin in Arabidopsis. Mol. Cell **5,** 1003–1011.

Goodman, C.D., Casati, P., and Walbot, V. (2004). A multidrug resistance-associated protein involved in anthocyanin transport in *Zea mays.* Plant Cell **16,** 1812–1826.

Greenberg, J.T., and Ausubel, F.M. (1993). Arabidopsis mutants compromised for the control of cellular damage during pathogenesis and aging. Plant J. **4,** 327–341.

Greenberg, J.T., and Yao, N. (2004). The role and regulation of programmed cell death in plant-pathogen interactions. Cell. Microbiol. **6,** 201–211.

Heath, M.C. (2000). Nonhost resistance and nonspecific plant defenses. Curr. Opin. Plant Biol. **3,** 315–319.

Heck, S., Grau, T., Buchala, A., Métraux, J.P., and Nawrath, C. (2003). Genetic evidence that expression of *NahG* modifies defense pathways independent of salicylic acid biosynthesis in the Arabidopsis-*Pseudomonas syringae* pv. *tomato* interaction. Plant J. **36,** 342–352.

Hernan, R., Fasheh, R., Calabrese, C., Frank, A.J., Maclean, K.H., Allard, D., Barraclough, R., and Gilbertson, R.J. (2003). ERBB2 up-regulates S100A4 and several other prometastatic genes in medulloblastoma. Cancer Res. **63,** 140–148.

Holt, B.F., Belkhadir, Y., and Dangl, J.L. (2005). Antagonistic control of disease resistance protein stability in the plant immune system. Science **309,** 929–932.

Hua, J., and Meyerowitz, E.M. (1998). Ethylene responses are negatively regulated by a receptor gene family in *Arabidopsis thaliana.* Cell **94,** 261–271.

Huitema, E., Vleeshouwers, V.G.A.A., Francis, D.M., and Kamoun, S. (2003). Active defense responses associated with non-host resistance of *Arabidopsis thaliana* to the oomycete pathogen *Phytophthora infestans.* Mol. Plant. Pathol. **4,** 487–500.

Jander, G., Norris, S.R., Rounsley, S.D., Bush, D.F., Levin, I.M., and Last, R.L. (2002). Arabidopsis map-based cloning in the post-genome era. Plant Physiol. **129,** 440–450.

Jasinski, M., Stukkens, Y., Degand, H., Purnelle, B., Marchand-Brynaert, J., and Boutry, M. (2001). A plant plasma membrane ATP binding cassette-type transporter is involved in antifungal terpenoid secretion. Plant Cell **13,** 1095–1107.

Jones, D.A., and Takemoto, D. (2004). Plant innate immunity: Direct and indirect recognition of general and specific pathogen-associated molecules. Curr. Opin. Immunol. **16,** 48–62.

Kang, L., Li, J.X., Zhao, T.H., Xiao, F.M., Tang, X.Y., Thilmony, R., He, S.Y., and Zhou, J.M. (2003). Interplay of the Arabidopsis nonhost resistance gene *NHO1* with bacterial virulence. Proc. Natl. Acad. Sci. USA **100,** 3519–3524.

Kanzaki, H., Saitoh, H., Ito, A., Fujisawa, S., Kamoun, S., Katou, S., Yoshioka, H., and Terauchi, R. (2003). Cytosolic HSP90 and HSP70 are essential components of INF1-mediated hypersensitive response and non-host resistance to *Pseudomonas cichorii* in *Nicotiana benthamiana.* Mol. Plant. Pathol. **4,** 383–391.

Kleffmann, T., Russenberger, D., von Zychlinski, A., Christopher, W., Sjolander, K., Gruissem, W., and Baginsky, S. (2004). The *Arabidopsis thaliana* chloroplast proteome reveals pathway abundance and novel protein functions. Curr. Biol. **14,** 354–362.

Kobayashi, Y., Yamada, M., Kobayashi, I., and Kunoh, H. (1997). Actin microfilaments are required for the expression of nonhost resistance in higher plants. Plant Cell Physiol. **38,** 725–733.

Kolling, R. (2002). Mutations affecting phosphorylation, ubiquitination and turnover of the ABC-transporter Ste6. FEBS Lett. **531,** 548–552.

Kuchler, K., Sterne, R., and Thorner, J. (1989). *Saccharomyces cerevisae STE6* gene product, a novel pathway for protein export in eukaryotic cells. EMBO J. **8,** 3973–3984.

Kushnir, S., Babiychuk, E., Storozhenko, S., Davey, M.W., Papenbrock, J., De Rycke, R., Engler, G., Stephan, U.W., Lange, H., Kispal, G., Lill, R., and Van Montagu, M. (2001). A mutation of the mitochondrial ABC transporter Sta1 leads to dwarfism and chlorosis in the *Arabidopsis* mutant *starik.* Plant Cell **13,** 89–100.

Lawton, K., Weymann, K., Friedrich, L., Vernooij, B., Uknes, S., and Ryals, J. (1995). Systemic acquired resistance in Arabidopsis requires salicylic acid but not ethylene. Mol. Plant Microbe Interact. **8,** 863–870.

Lee, H., Lee, K., Lee, J., Noh, E.W., and Lee, Y. (2005). AtPDR12 contributes to lead resistance in Arabidopsis. Plant Physiol. **138,** 827–836.

Liang, H., Yao, N., Song, L.T., Luo, S., Lu, H., and Greenberg, L.T. (2003). Ceramides modulate programmed cell death in plants. Genes Dev. **17,** 2636–2641.

Lipka, V., et al. (2005). Pre- and postinvasion defenses both contribute to nonhost resistance in Arabidopsis. Science **310,** 1180–1183.

Llorente, F., Alonso-Blanco, C., Sánchez-Rodríguez, C., Jorda, L., and Molina, A. (2005). ERECTA receptor-like kinase and heterotrimeric G protein from Arabidopsis are required for resistance to the necrotrophic fungus *Plectosphaerella cucumerina.* Plant J. **43,** 165–180.

Lukowitz, W., Gillmor, C.S., and Scheible, W.R. (2000). Positional cloning in Arabidopsis. Why it feels good to have a Genome Initiative working for you. Plant Physiol. **123,** 795–805.

Mach, J.M., Castillo, A.R., Hoogstraten, R., and Greenberg, J.T. (2001). The Arabidopsis-accelerated cell death gene *ACD2* encodes red chlorophyll catabolite reductase and suppresses the spread of disease symptoms. Proc. Natl. Acad. Sci. USA **98,** 771–776.

Maleck, K., Neuenschwander, U., Cade, R.M., Dietrich, R.A., Dangl, J.L., and Ryals, J.A. (2002). Isolation and characterization of broad-spectrum disease-resistant Arabidopsis mutants. Genetics **160,** 1661–1671.

Martinoia, E., Klein, M., Geisler, M., Bovet, L., Forestier, C., Kolukisaoglu, U., Muller-Rober, B., and Schulz, B. (2002). Multifunctionality of plant ABC transporters—More than just detoxifiers. Planta **214,** 345–355.

Masri, S.S., and Ellingboe, A.H. (1966). Primary infection of wheat and barley by *Erysiphe graminis.* Phytopathology **56,** 389–395.

Mellersh, D.G., and Heath, M.C. (2003). 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. Mol. Plant Microbe Interact. **16,** 398–404.

Meyer, S.L.F., and Heath, M.C. (1988). A comparison of the death induced by fungal invasion or toxic chemicals in cowpea epidermal cells. II. Responses induced by *Erysiphe cichoracearum.* Can. J. Bot. **66,** 624–634.

Moller, S.G., Kunkel, T., and Chua, N.H. (2001). A plastidic ABC protein involved in intercompartmental communication of light signaling. Genes Dev. **15,** 90–103.

Morris, K., Mackerness, S.A.H., Page, T., John, C.F., Murphy, A.M., Carr, J.P., and Buchanan-Wollaston, V. (2000). Salicylic acid has a role in regulating gene expression during leaf senescence. Plant J. **23,** 677–685.

Murashige, T., and Skoog, F. (1962). A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol. Plant. **15,** 473–497.

Muskett, P.R., Kahn, K., Austin, M.J., Moisan, L.J., Sadanandom, A., Shirasu, K., Jones, J.D.G., and Parker, J.E. (2002). *Arabidopsis* RAR1 exerts rate-limiting control of *R* gene-mediated defenses against multiple pathogens. Plant Cell **14,** 979–992.

Nawrath, C., and Métraux, J.P. (1999). Salicylic acid induction-deficient mutants of *Arabidopsis* express *PR-2* and *PR-5* and accumulate high levels of camalexin after pathogen inoculation. Plant Cell **11,** 1393–1404.

Neff, M.M., Neff, J.D., Chory, J., and Pepper, A.E. (1998). dCAPS, a simple technique for the genetic analysis of single nucleotide polymorphisms: Experimental applications in *Arabidopsis thaliana* genetics. Plant J. **14,** 387–392.

Nishimura, M.T., Stein, M., Hou, B.H., Vogel, J.P., Edwards, H., and Somerville, S.C. (2003). Loss of a callose synthase results in salicylic acid-dependent disease resistance. Science **301,** 969–972.

Nühse, T.S., Stensballe, A., Jensen, O.N., and Peck, S.C. (2004). Phosphoproteomics of the *Arabidopsis* plasma membrane and a new phosphorylation site database. Plant Cell **16,** 2394–2405.

Parker, J.E., Holub, E.B., Frost, L.N., Falk, A., Gunn, N.D., and Daniels, M.J. (1996). Characterization of *eds1*, a mutation in *Arabidopsis* suppressing resistance to *Peronospora parasitica* specified by several different *RPP* genes. Plant Cell **8,** 2033–2046.

Peart, J.R., et al. (2002). Ubiquitin ligase-associated protein SGT1 is required for host and nonhost disease resistance in plants. Proc. Natl. Acad. Sci. USA **99,** 10865–10869.

Petersen, M., et al. (2000). Arabidopsis MAP kinase 4 negatively regulates systemic acquired resistance. Cell **103,** 1111–1120.

Pighin, J.A., Zheng, H.Q., Balakshin, L.J., Goodman, I.P., Western, T.L., Jetter, R., Kunst, L., and Samuels, A.L. (2004). Plant cuticular lipid export requires an ABC transporter. Science **306,** 702–704.

Ramonell, K., Berrocal-Lobo, M., Koh, S., Wan, J.R., Edwards, H., Stacey, G., and Somerville, S. (2005). Loss-of-function mutations in chitin responsive genes show increased susceptibility to the powdery mildew pathogen *Erysiphe cichoracearum.* Plant Physiol. **138,** 1027–1036.

Ramonell, K.M., Zhang, B., Ewing, R.M., Chen, Y., Xu, D., Stacey, G., and Somerville, S. (2002). Microarray analysis of chitin elicitation in *Arabidopsis thaliana.* Mol. Plant. Pathol. **3,** 301–311.

Rate, D.N., Cuenca, J.V., Bowman, G.R., Guttman, D.S., and Greenberg, J.T. (1999). The gain-of-function *Arabidopsis acd6* mutant reveals novel regulation and function of the salicylic acid signaling pathway in controlling cell death, defenses, and cell growth. Plant Cell **11,** 1695–1708.

Rate, D.N., and Greenberg, J.T. (2001). The Arabidopsis *aberrant growth and death2* mutant shows resistance to *Pseudomonas syringae* and reveals a role for NPR1 in suppressing hypersensitive cell death. Plant J. **27,** 203–211.

Rhee, S.Y., Osborne, E., Poindexter, P.D., and Somerville, C.R. (2003). Microspore separation in the *quartet 3* mutants of Arabidopsis is impaired by a defect in a developmentally regulated polygalacturonase required for pollen mother cell wall degradation. Plant Physiol. **133,** 1170–1180.

Rozen, S., and Skaletsky, H.J. (2000). Primer3 on the WWW for general users and for biologist programmers. In Bioinformatics Methods and Protocols: Methods in Molecular Biology, S. Krawetz and S. Misener, eds (Totowa, NJ: Humana Press), pp. 365–386.

Saenz, G.S., and Taylor, J.W. (1999). Phylogeny of the Erysiphales (powdery mildews) inferred from internal transcribed spacer ribosomal DNA sequences. Can. J. Bot. **77,** 150–168.

Sasabe, M., Toyoda, K., Shiraishi, T., Inagaki, Y., and Ichinose, Y. (2002). cDNA cloning and characterization of tobacco ABC transporter: *NtPDR1* is a novel elicitor-responsive gene. FEBS Lett. **518,** 164–168.

Schaller, G.E., and Keiber, J.J. (September 1, 2001). Ethylene. In The Arabidopsis Book, C.R. Somerville and E.M. Meyerowitz, eds (Rockville, MD: American Society of Plant Biologists), doi/10.1199/tab.0071, http://www.aspb.org/publications/arabidopsis/.

Shah, J. (2003). The salicylic acid loop in plant defense. Curr. Opin. Plant Biol. **6,** 365–371.

Shih, M., Heinrich, P., and Goodman, H. (1991). Cloning and chromosomal mapping of nuclear genes encoding chloroplast and cytosolic glyceraldehyde-3-phosphate-dehydrogenase from *Arabidopsis thaliana.* Gene **104,** 133–138.

Staswick, P., Su, W., and Howell, S. (1992). Methyl-jasmonate inhibition of root growth and induction of a leaf protein are decreased in an *Arabidopsis thaliana* mutant. Proc. Natl. Acad. Sci. USA **89,** 6837–6840.

Stukkens, Y., Bultreys, A., Grec, S., Trombik, T., Vanham, D., and Boutry, M. (2005). *NpPDR1*, a pleiotropic drug resistance-type ATP-binding cassette transporter from *Nicotiana plumbaginifolia*, plays a major role in plant pathogen defense. Plant Physiol. **139,** 341–352.

Sturaro, M., Hartings, H., Schmelzer, E., Velasco, R., Salamini, F., and Motto, M. (2005). Cloning and characterization of *GLOSSY1*, a maize gene involved in cuticle membrane and wax production. Plant Physiol. **138,** 478–489.

Thordal-Christensen, H. (2003). Fresh insights into processes of non-host resistance. Curr. Opin. Plant Biol. **6,** 351–357.

Tsuji, J., Jackson, E.P., Gage, D.A., Hammerschmidt, R., and Somerville, S.C. (1992). Phytoalexin accumulation in *Arabidopsis thaliana* during the hypersensitive reaction to *Pseudomonas syringae* pv. *syringae.* Plant Physiol. **98,** 1304–1309.

van den Brule, S., and Smart, C.C. (2002). The plant PDR family of ABC transporters. Planta **216,** 95–106.

van Wees, S.C.M., and Glazebrook, J. (2003). Loss of non-host resistance of Arabidopsis *NahG* to *Pseudomonas syringae* pv. *phaseolicola* is due to degradation products of salicylic acid. Plant J. **33,** 733–742.

Vogel, J., and Somerville, S. (2000). Isolation and characterization of powdery mildew-resistant Arabidopsis mutants. Proc. Natl. Acad. Sci. USA **97,** 1897–1902.

Vogel, J.P., Raab, T.K., Schiff, C., and Somerville, S.C. (2002). *PMR6*, a pectate lyase-like gene required for powdery mildew susceptibility in *Arabidopsis.* Plant Cell **14,** 2095–2106.

Vogel, J.P., Raab, T.K., Somerville, C.R., and Somerville, S.C. (2004). Mutations in *PMR5* result in powdery mildew resistance and altered cell wall composition. Plant J. **40,** 968–978.

Xie, D.X., Feys, B.F., James, S., Nieto-Rostro, M., and Turner, J.G. (1998). *COI1*: An Arabidopsis gene required for jasmonate-regulated defense and fertility. Science **280,** 1091–1094.

Yun, B.W., Atkinson, H.A., Gaborit, C., Greenland, A., Read, N.D., Pallas, J.A., and Loake, G.J. (2003). Loss of actin cytoskeletal function and EDS1 activity, in combination, severely compromises non-host resistance in Arabidopsis against wheat powdery mildew. Plant J. **34,** 768–777.

Zimmerli, L., Jakab, C., Métraux, J.P., and Mauch-Mani, B. (2000). Potentiation of pathogen-specific defense mechanisms in Arabidopsis by beta-aminobutyric acid. Proc. Natl. Acad. Sci. USA **97,** 12920–12925.

Zimmerli, L., Stein, M., Lipka, V., Schulze-Lefert, P., and Somerville, S. (2004). Host and non-host pathogens elicit different jasmonate/ethylene responses in Arabidopsis. Plant J. **40,** 633–646.

Zimmermann, P., Hirsch-Hoffmann, M., Hennig, L., and Gruissem, W. (2004). GENEVESTIGATOR. Arabidopsis microarray database and analysis toolbox. Plant Physiol. **136,** 2621–2632.

NOTE ADDED IN PROOF

Kobae et al. (2006) report that *pdr8*/*pen3* mutants are compromised in nonhost resistance to *Phytophthora infestans* but show increased resistance to *Pseudomonas syringae* pv tomato DC3000.

Kobae, Y., Sekino, T., Yoshioka, H., Nakagawa, T., Martinoia, E., and Maeshima, M. (January 13, 2006). Loss of AtPDR8, a plasma membrane ABC transporter of *Arabidopsis thaliana*, causes hypersensitive cell death upon pathogen infection. Plant Cell Physiol. http://dx.doi.org/10.1093/pcp/pcj001.