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# AGB1 and PMR5 Contribute to PEN2-Mediated Preinvasion Resistance to *Magnaporthe oryzae* in *Arabidopsis thaliana*

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**Rice blast, caused by *Magnaporthe oryzae*, is a devastating disease of rice (*Oryza sativa*). The mechanisms involved in resistance of rice to blast have been studied extensively and the rice–*M. oryzae* pathosystem has become a model for plant–microbe interaction studies. However, the mechanisms involved in nonhost resistance (NHR) of other plants to rice blast are still poorly understood. Here, we investigated interactions between *Arabidopsis thaliana* and *M. oryzae* to identify the genetic basis of NHR. In *A. thaliana* accessions, preinvasion resistance to *M. oryzae* in Col-0 was stronger than that of L*er*. To examine the genetic basis underlying the natural variation in the responses, we used a well-established set of recombinant inbred lines (RIL) derived from a Col × L*er* cross and identified three quantitative trait loci that govern the expression of NHR in *A. thaliana* against *M. oryzae*. Among the *penetration* (*pen*) mutants, only the *pen2* mutant allowed increased penetration into epidermal cells by *M. oryzae*. Double mutant analysis indicated that AGB1 and PMR5 contribute to PEN2-mediated preinvasion resistance to *M. oryzae* in *A. thaliana*, suggesting a complex genetic network regulating the resistance. Our results demonstrate that *A. thaliana* can be used to study mechanisms of NHR to *M. oryzae*.**

Rice is a staple crop of economic importance in many countries. One of the most serious and widespread diseases of rice is blast, caused by the ascomycete fungus *Magnaporthe oryzae*. The mechanisms involved in resistance of rice to blast have been studied extensively and the rice–*M. oryzae* pathosystem has become a model for the study of plant–microbe interactions because both whole-genome sequences and functional genomic approaches are available (Koga 2001; Dean et al. 2005; Ebbole 2007; Kankanala et al. 2007; Wilson and Talbot 2009). The infection of rice by *M. oryzae* follows a developmental process that has been observed in many foliar fungal pathogens. A germ tube produced from the conidium differentiates into a specialized infectious structure called the appressorium, which adheres tightly to the plant surface using mucilage. The fungus generates massive turgor pressure inside the melanized appressorium and a thin penetration peg pierces the host surface, using this pressure to enter a leaf epidermal cell (Howard et al. 1991). After penetration, the peg differentiates into bulbous and lobed infectious hyphae that grow intracellularly and intercellularly.

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Most plants are immune to the majority of would-be pathogens and susceptible to only a relatively small number of adapted microbes. Consequently, disease is the exception and not the rule. Disease resistance shown by an entire plant species to all genetic variants of a nonadapted pathogen species is the most common form of plant immunity and termed nonhost resistance (NHR) (Heath 2000; Lipka et al. 2008). Although NHR represents the most common and durable form of plant resistance in nature, it has thus far been poorly understood at the molecular level.

Recently, *Arabidopsis thaliana* mutants with altered nonhost interactions upon *Blumeria graminis hordei* infection were identified and three genes—*PENETRATION 1* (*PEN1*), *PEN2,* and *PEN3*—were identified (Collins et al. 2003; Lipka et al. 2005; Kobae et al. 2006; Stein et al. 2006). Single mutants of these genes exhibit several-fold increased invasion frequencies by *B. graminis hordei* but no increase in overall susceptibility owing to concomitant hypersensitive response (HR)-like cell death of invaded epidermal cells. *PEN1* encodes a plasma membrane-anchored syntaxin with a soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) domain (Collins et al. 2003). SNARE-domain-containing proteins are key players in vesicle-associated membrane fusion and secretion processes, including exocytosis and endocytosis. *PEN2* encodes 1 of 48 predicted *A. thaliana* family 1 glycoside hydrolases (F1GHs) (Lipka et al. 2005). Recently, it has been found that PEN2 is an atypical myrosinase (a type of β-thioglucoside glycohydrolase), which is involved in a glucosinolate metabolism in defense responses (Bednarek et al. 2009; Clay et al. 2009). *PEN3* encodes one of the 15 pleiotropic drug resistance (PDR) ATP binding cassette (ABC) transporters present in the *A. thaliana* genome (Kobae et al. 2006; Stein et al. 2006). These are ubiquitous transmembrane proteins that function in the ATP-dependent transport of a wide variety of substrates across extracellular and intracellular membranes. *A. thaliana* mutants showing enhanced entry are still nonhost plants for nonadapted powdery mildews owing to effective postentry cell death. Systematic analyses with multiple mutant combinations revealed that posthaustorial NHR requires enhanced susceptibility 1 (EDS1), phytoalexin-deficient 4 (PAD4), and senescence-associated gene 101 (SAG101) (Lipka et al. 2005). Single mutants for *EDS1*, *PAD4*, and *SAG101* have no or only a minor effect on preinvasion resistance to powdery mildews. However, *pen2-pad4-sag101* triple mutants are fully susceptible for the nonadapted pea powdery mildew and allow the monocot pathogen *B. graminis hordei* to establish basic compatibility (Lipka et al. 2005). Thus, *A. thaliana* NHR to nonadapted biotrophic powdery mildews is based upon two successive, multicomponent, and independently effective de-

fense layers: PEN-mediated preinvasion resistance and postinvasion immunity, controlled by EDS1, PAD4, and SAG101 (Lipka et al. 2005). In addition, NHR of *A. thaliana* to other pathogens has also been studied (Huitema 2003; Yun et al. 2003; Shimada et al. 2006; Shafiei 2007; Loehrer et al. 2008).

The involvement of well-described defense-related genes has been investigated in NHR. The salicylic acid (SA) signal transduction pathway plays an important role in defense responses initiated by resistance (*R*) genes. However, its contribution to NHR is less clear. *A. thaliana* plants expressing *NahG*, which converts SA to catechol (Lawton et al. 1995), do not accumulate SA and are compromised in NHR to some but not all bacterial and fungal pathogens (Lu et al. 2001; Mellersh and Heath 2003; Van Wees and Glazebrook 2003; Yun et al. 2003). The jasmonate and ethylene (JA/ET) pathway has also been tested for its role in NHR. In *A. thaliana*, blocks in the JA or ET pathways generally did not promote enhanced susceptibility to nonhost pathogens (Mellersh and Heath 2003; Yun et al. 2003). Heterotrimeric G protein complexes couple extracellular signals to downstream effectors via cell surface receptors. Although recent studies have shown that the *A. thaliana* heterotrimeric G proteins are involved in defense responses against several pathogens (Suharsono et al. 2002; Trusov et al. 2006, 2008; Ishikawa 2009), their contribution to NHR is less clear. Specific host genes or proteins termed compatibility or susceptibility factors are believed to be essential for successful pathogenesis by a given microbe, and a lack of these factors is predicted to result in resistance to an otherwise virulent pathogen (Jones and Dangl 2006). In a screen to recover loss-of-susceptibility to *Golovinomyces cichoracearum* mutants in *A. thaliana*, the *powdery mildew resistant* (*pmr*) mutants *pmr1* to *pmr6* were isolated and some of the genes have been cloned (Vogel and Somerville 2000). *PMR2* is allelic to *MLO2* (Consonni et al. 2006), *PMR4* encodes a wound- and pathogen-associated callose synthase (Jacobs et al. 2003; Nishimura et al. 2003), *PMR5* belongs to a large family of plant-specific genes of unknown function (Vogel et al. 2004), and *PMR6* encodes a glycosyl-phosphatidyl-inositol (GPI)-anchored pectate-lyase-like protein (Vogel et al. 2002). However, their contribution to NHR is poorly understood.

Recently, Faivre-Rampant and associates (2008) have initiated the characterization of NHR in rice challenged with *M. grisea* strains isolated from other monocot species and compared it with compatible or incompatible interactions of rice with strains of the *M. oryzae* species isolated from wheat and rice. However, the mechanisms involved in NHR of nonhost plants to rice blast are still poorly understood, although the cytological description of events occurring during a rice blast infection on these plants was investigated as early as the 1940s (Yoshii 1949; Hashioka and Kusadome 1975a,b). Here, we present the interactions of *A. thaliana* with *M. oryzae* as a model pathosystem to dissect out plant resistance mechanisms in *A. thaliana.*

## RESULTS

### Interaction between *A. thaliana* and *M. oryzae*.

The hemibiotrophic ascomyceteous fungus *M. oryzae*, which belongs to the *M. grisea* species, includes the rice blast pathogen that has a devastating effect on rice (*Oryza sativa*). The blast fungus mechanically breaks the plant surface using an appressorium, a dome-shaped cell that generates massive turgor pressure. The appressorium produces a specialized hypha, a penetration peg, which pierces the plant surface (Koga 2001; Kankanala et al. 2007; Wilson and Talbot 2009). In this study, *A. thaliana* accessions were tested for their response to *M. oryzae* and interaction phenotypes were assessed at the cellular level (Fig. 1 and data not shown). Leaves of infected *A. thaliana* plants were harvested at 3 days postinoculation (dpi) and examined microscopically. Upon inoculation onto the Col-0 accession of *A. thaliana*, conidia of *M. oryzae* germinated and formed appressoria at the tips of germ tubes. The majority (approximately 99.9%) of conidia failed to penetrate leaf epidermal cells and fungal growth terminated during penetration attempts (Fig. 1E). No visible cellular responses, such as the HR-like cell death, occurred in the attacked epidermal cell (Fig. 1A and C). Thus, a block at the penetration step was the most prevalent form of resistance to *M. oryzae* in Col-0. In contrast, some *M. oryzae* conidia (approximately 5%) penetrated into epidermal cells of the L*er* accession of *A. thaliana* (Fig. 1E). The penetrated epidermal cells accumulated autofluorescent compounds, reported as being a reliable marker for the HR-like cell death (Koga 1994) (Fig. 1B and D). Thus, a block at the postpenetration step is also important for resistance to *M. oryzae* in *A. thaliana*. We also tested other *A. thaliana* accessions (Ksk-1, No-0, and RLD-1) for their response to *M. oryzae*. All the accessions tested exhibited a level of resis-

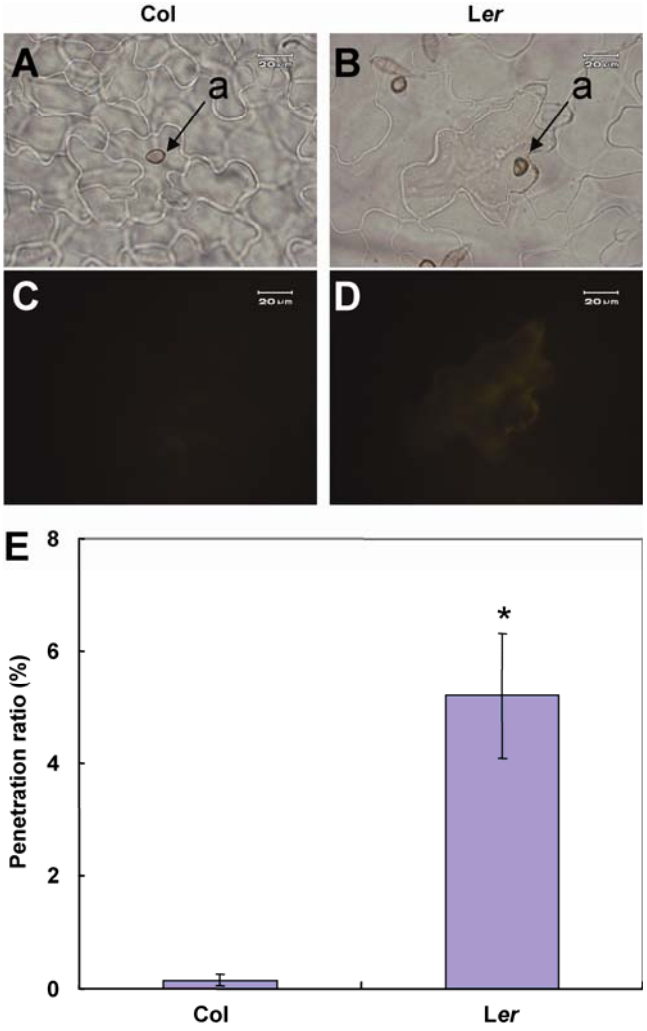


**Fig. 1.** Plant responses to attempted *Magnaporthe oryzae* infection. **A** and **B,** Light microscopic view of infection sites of **A,** Col-0 plants and **B,** L*er* plants at 3 days postinoculation (dpi); a = appressorium. **C** and **D,** Epidermal cell-death-associated autofluorescence of *M. oryzae*-inoculated leaves of **C,** Col-0 plants and **D,** L*er* plants at 3 dpi was viewed by fluorescence microscopy. **E,** Mean of the frequency of *M. oryzae* penetration on *Arabidopsis thaliana* at 3 dpi, expressed as a percentage of total appressoria. Data were collected from six leaves from six independent plants of each accession. A minimum of 100 infection sties was inspected per leaf. Results represent the mean ± standard error of three independent experiments. Asterisks indicate a significant difference from Col-0 ($P < 0.05$; Student's *t* test). Bars = 20 μm.

tance to penetration similar to that of the Col-0 accession (data not shown). These findings support the idea that *M. oryzae* is a nonhost pathogen of *A. thaliana*.

**Identification of quantitative trait loci for NHR in *A. thaliana* against *M. oryzae*.**

To examine the genetic basis of the variations in the responses of the different accessions of *A. thaliana* to *M. oryzae,* we used a well-established set of recombinant inbred lines (RIL), derived from a Col × L*er* cross (Lister and Dean 1993). This resource facilitates the rapid evaluation of genetic variation and offers the ability to map any quantitative trait loci (QTL) controlling this variability (Ikka et al. 2007; Shafiei 2007). The Col/L*er* RIL were tested for variation in their response to *M. oryzae*. Leaves of infected RIL were harvested at 3 dpi and examined microscopically. A population of 98 RIL was scored for resistance to penetration by *M. oryzae.* Using the composite interval mapping (CIM) method with QTL Cartographer software (Basten et al. 1994), we analyzed the QTL that controlled penetration resistance of the Col/L*er* RIL against *M. oryzae.* Three QTL ($P < 0.05$) were identified on chromosomes 1 and 2 (Fig. 2). QTL1 was positioned at 50.8 centimorgans (cM) on chromosome 1 and had a logarithm of odds (LOD) score of 4.50, which accounted for 15.0% of the total variation for this trait. QTL2 was positioned at 40.4 cM on chromosome 2 and had an LOD score of 4.32, which accounted for 14.0% of the total variation for this trait. QTL3 was positioned at 49.5 cM on chromosome 2 and had an LOD score of 3.14, which accounted for 10.0% of the total variation for this trait. Collectively, these QTL for penetration resistance represented 39.0% of the total variation.

**Response of *A. thaliana* defense-related mutants to *M. oryzae* infection.**

To determine whether any of the known plant defense pathways affected the *A. thaliana–M. oryzae* interactions, the following *A. thaliana* mutants and transgenic plants were inoculated with *M. oryzae* and monitored by microscopy: *npr1-1* (Cao et al. 1994); *sid2-1* (Nawrath and Metraux 1999); *pad1-1, pad2-1, pad3-1, pad4-1*, and *pad5-1* (Glazebrook and Ausubel 1994); *pmr1-1, prm2-1, pmr3-1, pmr4-1, pmr5-1*, and *pmr6-1* (Vogel and Somerville 2000); *Atmlo2-7* (SALK_079850); *eds1-2* (L*er*) (Aarts et al. 1998); *eds2-1, eds3-1, eds4-1, eds5-1, eds6-1, eds7-1, eds8-1, eds9-1, eds10-1, eds11-1, eds12-1, eds13-1, eds14-1, eds15-1*, and *eds16-1* (Glazebrook et al. 1996); *ein2-1* (Alonso et al. 1999); *coi1-1* (Xie et al. 1998); *jar1-1* (Staswick et al. 1992); *gpa1-4* and *agb1-2* (Chen et al. 2006); *rar1-21* (Tornero et al. 2002); *edm1-1* (Tor et al. 2002); *nho1-1* (Lu et al. 2001); *ndr1-1* (Century et al. 1995); and *NahG* (Lawton et al. 1995) (all Col-0 background except as noted). All these mutants exhibited a level of penetration resistance similar to that of the wild-type plants (data not shown), suggesting that none of these mutants significantly compromised resistance to *M. oryzae.*

Recently, the identification of *A. thaliana* mutants that are compromised in penetration resistance against the barley powdery mildew pathogen, *B. graminis hordei*, has led to significant insights into this mechanism. Mutations in *PEN* genes have highlighted possible roles for vesicle trafficking, antimicrobial compounds, and extracellular transport of small molecules in NHR against *B. graminis hordei* (Collins et al. 2003; Lipka et al. 2005; Kobae et al. 2006; Stein et al. 2006). We tested *pen* mutants (*pen1*, *pen2*, and *pen3*) in resistance against *M. oryzae.* Of these, only *pen2* had a significantly increased penetration ratio compared with the wild type (Fig. 3A). Upon inoculation onto *pen2* plants, *M. oryzae* conidia germinated and produced appressoria which attempted penetration of the epidermal cell in an infection process similar to that which occurs on its host (rice) (Koga 2001). Some of them could penetrate leaf epidermal cells. This led to the accumulation of autofluorescent compounds, callose, and $H_2O_2$ in the

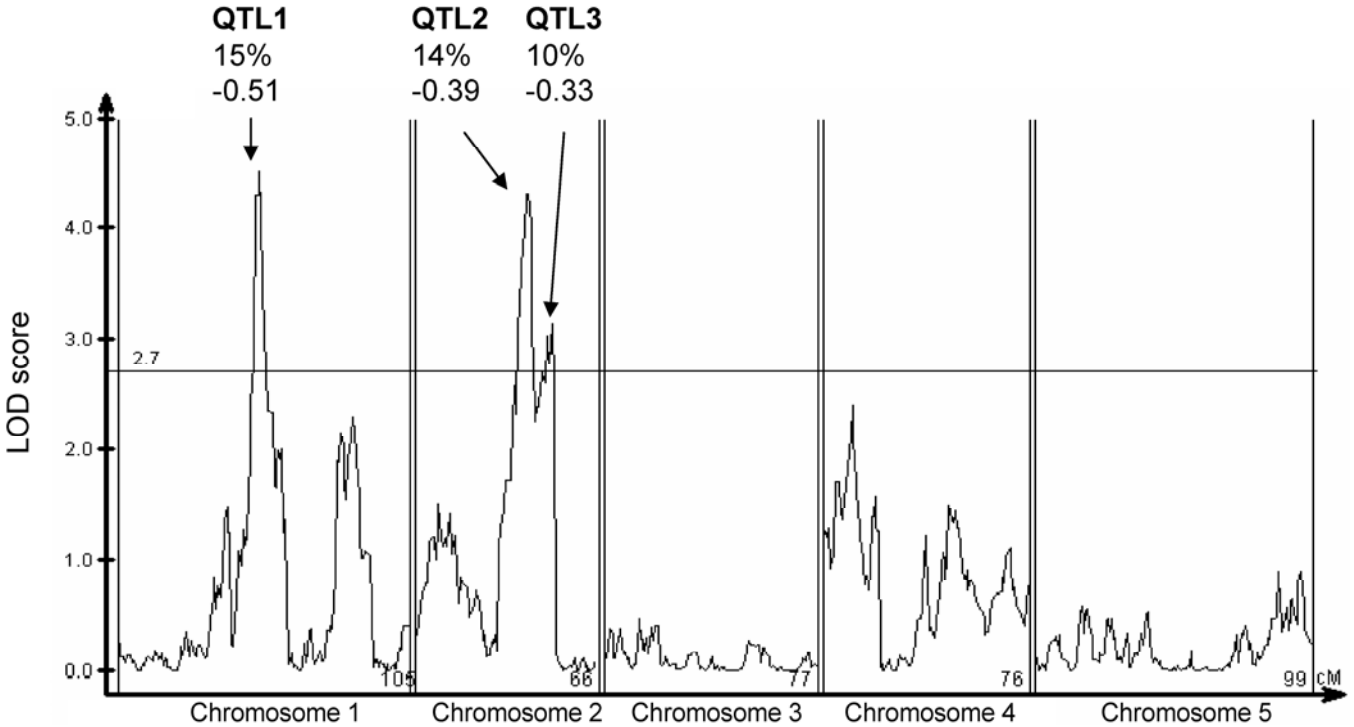


**Fig. 2.** Logarithm of odds (LOD) score profile for preinvasion resistance in a Col/L*er* recombinant inbred line population. LOD score curves are shown on the vertical scales and horizontal axes show map distances in centimorgans. Chromosome numbers are indicated in the panel. The 2.7 LOD threshold used for quantitative trait locus (QTL) detection is shown as a horizontal line. Percentage of the total phenotypic variance explained and the allele additive effect of each QTL is shown. A negative or positive sign for additive effect indicates that the given QTL was derived from either the Col or the L*er* accession, respectively.

challenged epidermal cell, which preceded the HR-like cell death (Fig. 3B through F). In the interaction, some fungi formed intracellular infection hyphae but fungal growth terminated during elongation attempts (Fig. 3G). *M. oryzae* infection did not progress beyond this stage. Thus, loss of PEN2 function does not allow *M. oryzae* to complete its life cycle and form asexual conidia on *A. thaliana* as it does on rice, suggesting that additional factors contribute to NHR to *M. oryzae* in *A. thaliana*.

**Double-mutant analysis to evaluate the role of defense signaling pathways in the NHR to *M. oryzae*.**

The results described above support a crucial role for PEN2 in controlling the penetration resistance of *A. thaliana* to *M. oryzae.* As a next step, double mutants were generated between *pen2* and defense-related mutants or transgenic plants to evaluate the role of well-described defense signaling pathways in the resistance to *M. oryzae* in a *pen2* background. Leaves of infected *A. thaliana* plants were harvested at 3 dpi and examined microscopically. First, we investigated double mutants affecting the SA pathway (*pen2 pad4, pen2 eds5, pen2 sid2,* and *pen2 NahG*). Among them, only *pen2 NahG* plants showed significant increases in penetration ratio relative to *pen2* plants (Fig 4). NahG-expressing plants convert SA to catechol. The SA biosynthetic mutant *sid2* did not affect a penetration phenotype of *pen2*; therefore, partial loss of penetration resistance might be due to NahG-dependent catechol production. However, no significant difference in penetration was detected between *pen2* and *pen2* treated with catechol (data not shown). Second, we investigated double mutants affecting the JA/ET pathway (*pen2 jar1, pen2 coi1,* and *pen2 ein2*). Although *pen2 jar1* and *pen2 coi1* plants allowed slightly increased frequency of penetration, none of these double mutants showed significant increases in penetration ratio (Fig. 5) compared with *pen2* plants. These results suggest that neither the SA nor the JA/ET signal transduction pathways contribute significantly to penetration resistance to *M. oryzae* in *A. thaliana.* Third, we investigated double mutants affected in G protein signaling (*pen2 gpa1* and *pen2 agb1*). Although *pen2 gpa1* plants tended to have decreased penetration ratio compared with *pen2* plants, *pen2 agb1* plants had a significantly increased penetration ratio compared with *pen2* plants, suggesting involvement of AGB1 in the penetration resistance (Fig. 5). In *pen2 NahG* and *pen2 agb1* plants, *M. oryzae* penetrated the epidermal cell but did not grow further, either inter- of intracellularly, into neighboring cells (data not shown).

**Double mutant analysis to evaluate the role of *PMR* genes on NHR to *M. oryzae*.**

*A. thaliana pmr* mutants are loss-of-function mutants that provide enhanced disease resistance to the powdery mildew, *G.*

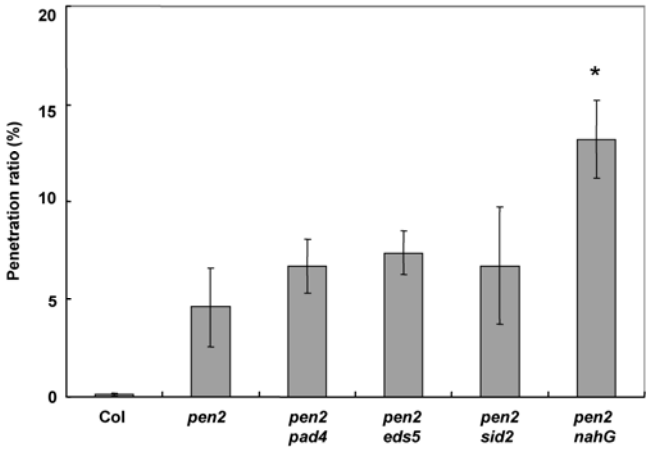


**Fig. 4.** Double mutant analysis to evaluate the role of the salicylic acid (SA) signal transduction pathway on resistance to *Magnaporthe oryzae* in *Arabidopsis thaliana*. Quantitative analysis of cell entry on SA signal transduction mutants in a *penetration* (*pen*)*2* background. Mean of the frequency of *M. oryzae* penetration on double mutants at 3 days postinoculation was expressed as a percentage of total appressoria. Data were collected from six leaves from six independent plants per line. A minimum of 100 infection sties was inspected per leaf. Results represent mean ± standard error of three independent experiments. Asterisks indicate a significant difference from *pen2* ($P < 0.05$; Student's *t* test).

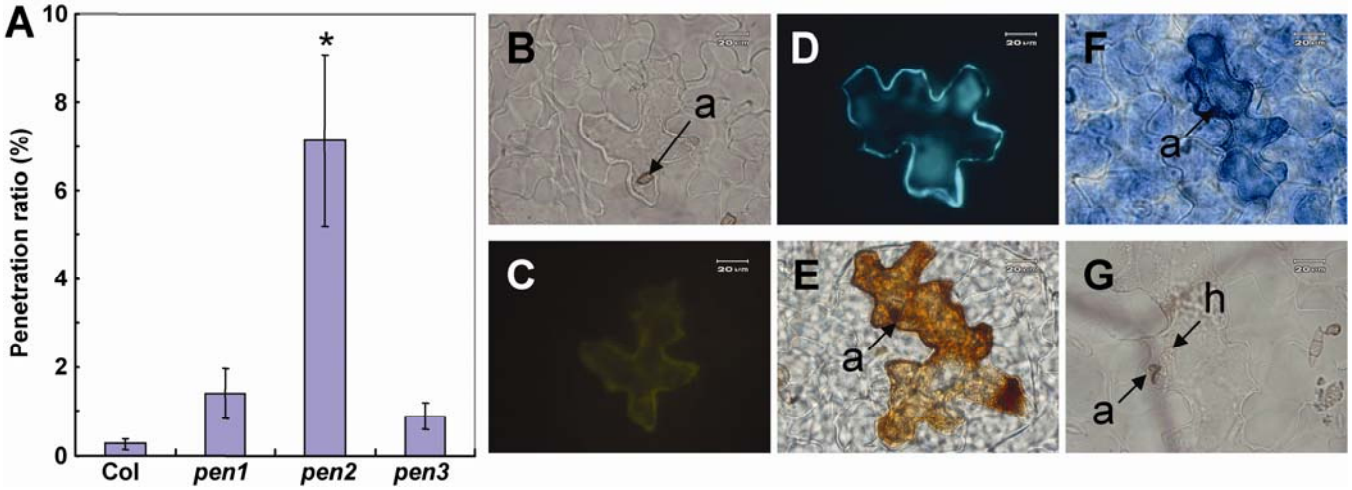


**Fig. 3.** *penetration* (*pen*)*2* resistance phenotypes. **A,** Quantitative analysis of cell entry on *pen* mutants. Mean of the frequency of *Magnaporthe oryzae* penetration on *pen* mutants at 3 days postinoculation (dpi) was expressed as a percentage of total appressoria. Data were collected from six leaves from six independent plants per line. A minimum of 100 infection sties was inspected per leaf. Results represent the mean ± standard error of three independent experiments. Asterisks indicate a significant difference from Col-0 ($P < 0.05$; Student's *t* test). **B,** Light microscopic view of infection sites of *pen2* plants at 3 dpi; a = appressorium. **C,** Epidermal cell-death-associated autofluorescence at infection site of *pen2* plants at 3 dpi was viewed by fluorescence microscopy. **D,** Callose deposition at infection site of *pen2* plants at 3 dpi was visualized by fluorescence microscopy following aniline blue staining. **E,** $H_2O_2$ accumulation at infection site of *pen2* plants at 3 dpi was monitored by 3,3-diaminobenzideine tetrahydrochloride staining by bright-field microscopy; a = appressorium. **F.** Cell death at infection site of *pen2* plants at 3 dpi was visualized by trypan blue staining by bright-field microscopy; a = appressorium. **G.** Intracellular infection hyphae inside infected epidermal cell of *pen2* plants at 3 dpi; a = appressorium and h = hyphae. Bars = 20 μm.

*cichoracearum* (Vogel and Somerville 2000). *PMR2* is allelic to *MLO2* (Consonni et al. 2006), *PMR4* encodes a wound- and pathogen-associated callose synthase (Jacobs et al. 2003; Nishimura et al. 2003), *PMR5* belongs to a large family of plant-specific genes of unknown function (Vogel et al. 2004), and *PMR6* encodes a GPI-anchored pectate-lyase-like protein (Vogel et al. 2002). However, their contribution to NHR is poorly understood. Because the single *pmr* mutations did not affect the penetration resistance to *M. oryzae* (data not shown), double mutants were generated between *pen2* and *pmr* mutants to determine whether these factors were necessary for the resistance to *M. oryzae* in a *pen2* background. Leaves of infected *A. thaliana* plants were harvested at 3 dpi and examined microscopically. Only *pen2 pmr5* plants showed significant increases in penetration ratio compared with *pen2* plants (Fig. 6). This indicates that PMR5 has a function in preinvasion resistance against *M. oryzae*. In *pen2 pmr5* plants, *M. oryzae* penetrated the epidermal cell but did not grow further as in the case of *pen2 NahG* and *pen2 agb1* plants (data not shown).

We also investigated callose formation, including papillary-associated callose and whole-cell callose, in *pen2 pmr* epidermal cells upon inoculation with *M. oryzae* by aniline blue staining. Papillary-associated callose formation beneath fungal appressoria is a widespread plant response in their interaction with diverse fungal parasites, and whole-cell callose is a marker for the HR-like cell death (Koga 1994; Jacobs et al. 2003; Nishimura et al. 2003). UV-induced fluorescence with aniline blue was readily detectable in plant cells beneath *M. oryzae* appressoria (papillary-associated callose) (Fig. 7A through L) and invaded epidermal cells (whole-cell callose) (Fig. 7M through X). The papillary-associated callose production beneath *M. oryzae* appressoria in wild-type plants suggests that *A. thaliana* epidermal cells detect and respond to entry attempts of the fungus. However, whole-cell callose formation was detected only in the *pen2* background, except for *pen2 pmr4*, suggesting that penetration of the fungus in the cells is required for the production of callose. Because *pmr4* is specifically impaired in the accumulation of callose, fluorescence was very weak in the *pen2 pmr4* double mutant compared with *pen2* plants (Fig. 7H and T). Furthermore, the callose accumulation in *pen2 pmr5* plants was the same as in *pen2* plants (Fig. 7J and V). These results suggest that callose accumulation is dispensable for pre- and postinvasion resistance to *M. oryzae* in *A. thaliana*.

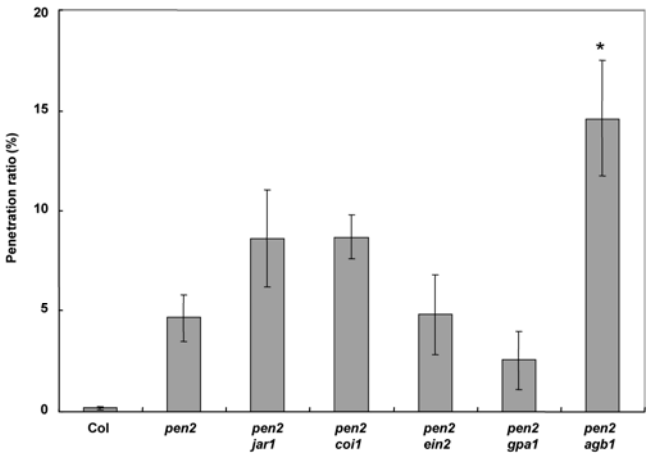


**Fig. 5.** Double mutant analysis to evaluate the role of the jasmonate and ethylene signal transduction pathway and heterotrimeric G protein on resistance to *Magnaporthe oryzae* in *Arabidopsis thaliana*. The frequency of *M. oryzae* penetration on double mutants at 3 days postinoculation was expressed as a percentage of total appressoria. Data were collected from six leaves from six independent plants per line. A minimum of 100 infection sties was inspected per leaf. Results represent mean ± standard error of three independent experiments. Asterisks indicate a significant difference from *pen2* ($P < 0.05$; Student's *t* test).

## DISCUSSION

Among *pen* mutants, only the *pen2* mutant allowed increased penetration into epidermal cells by the nonadapted pathogen *M. oryzae*. This indicates that the effect of *pen2* is broader than that of *pen1* (which affects only *B. graminis hordei* infection), as described previously (Lipka et al. 2005). We have also shown that NahG, AGB1, and PMR5 contribute to the PEN2-mediated preinvasion resistance in the *A. thaliana–M. oryzae* interactions, suggesting a complex genetic network regulating the resistance.

Analysis of a series of well-characterized defense-response mutants following *M. oryzae* challenge revealed that none of these lines was significantly more susceptible to *M. oryzae* than the wild type (data not shown). Thus, the signaling pathways in which these mutants are affected, including SA- and JA/ET-dependent pathways, are likely either to function redundantly or to be dispensable for resistance against *M. oryzae.*

In interactions with nonadapted powdery mildews, PEN1 is recruited in the plasma membrane microdomain beneath the powdery mildew appressoria (Collins et al. 2003). Cell-wall appositions formation (papillae) is delayed in response to attempted powdery mildew entry in *pen1* plants, and this correlates with impaired preinvasion resistance (Collins et al. 2003; Assaad et al. 2004). However, *M. oryzae* failed to invade epidermal cells of *pen1* plants (Fig. 3). Although *ROR2* is the functional homolog of *A. thaliana PEN1* and is required for *mlo*-mediated broad-spectrum resistance against all tested host barley powdery mildew isolates (Collins et al. 2003), *ROR2* is not essential for resistance against strains of the rice blast fungus *M. oryzae* that can infect barley (Jarosch et al. 2005). Thus, these data suggest that PEN1/ROR2 may not exert an entry-limiting function in preinvasion resistance to *Magnaporthe* spp. in plants.

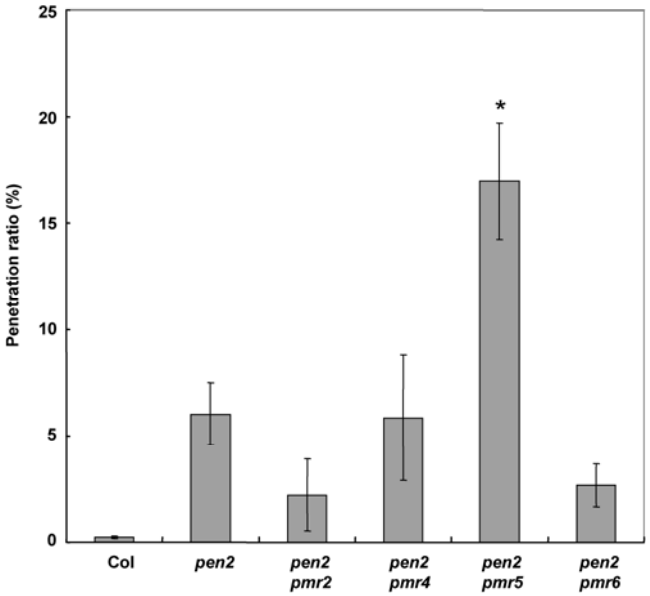


**Fig. 6.** Double mutant analysis to evaluate the role of the *powdery mildew resistant* (*PMR*) genes on resistance to *Magnaporthe oryzae* in *Arabidopsis thaliana*. The frequency of *M. oryzae* penetration on double mutants at 3 days postinoculation was expressed as a percentage of total appressoria. Data were collected from six leaves from six independent plants per line. A minimum of 100 infection sties was inspected per leaf. Results represent mean ± standard error of three independent experiments. Asterisks indicate a significant difference from *pen2* ($P < 0.05$; Student's *t* test).

Stein and associates (2006) have proposed a model for the role of PEN3 in penetration resistance 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. In this model, PEN3 exports toxic secondary metabolites to the apoplast at sites of attempted invasion and the myrosinase PEN2 activates one of these toxins by hydrolysis of a nontoxic precursor metabolite. Because preinvasion resistance to *M. oryzae* is PEN2 dependent but PEN3 independent (Fig. 3A), PEN2 might activate other toxins by hydrolysis of other precursor metabolites, and an ABC transporter protein other than PEN3 might export it to the apoplast in the *A. thaliana–M. oryzae* interactions.

Although NHR to *M. oryzae* was not compromised in *NahG* transgenic plants (data not shown), *pen2 NahG* plants allowed increased entry into epidermal cells by *M. oryzae* compared with *pen2* plants (Fig. 4). Because *pen2 sid2* plants did not provide evidence of the involvement of the SA pathway in NHR (Fig. 4), it is likely that SA-independent responses mediated by NahG contribute to NHR. Although NahG affects the functioning of the SA pathway, it also exerts an SA-independent, yet-to-be-characterized effect on resistance (Van Wees and Glazebrook 2003). NahG-expressing plants convert SA to catechol; therefore, partial loss of penetration resistance might be due to NahG-dependent catechol production (Van Wees and Glazebrook 2003). In fact, NahG-dependent catechol production affects *Atmlo2* resistance against powdery mildew in *Atmlo2 NahG* plants (Consonni et al. 2006). However, no significant difference in penetration following *M. oryzae* challenge was detected between *pen2* and *pen2* treated with catechol (data not shown). Thus, enhanced *M. oryzae* epidermal cell entry in *pen2 NahG* plants is likely due to non-SA-dependent responses associated with *NahG* expression but not catechol production. SA-independent effects of *NahG* have been reported previously (Heck et al. 2003; Brodersen et al. 2005; Zhang 2008).

The *pmr* mutants were isolated in a screen to recover loss-of-susceptibility to *G. cichoracearum* mutants in *A. thaliana*, and some of the genes have been cloned. *PMR2* encodes an ortholog of the barley MILDEW LOCUS O (MLO), MLO2 (Consonni et al. 2006). Both barley MLO and *A. thaliana* PMR2 (MLO2) serve as functionally conserved negative regulators of entry resistance to adapted obligate biotrophic powdery mildews (Buschges et al. 1997; Consonni et al. 2006). Interestingly, loss of MLO reduces preinvasion resistance to *M. oryzae* in barley (Jarosch et al. 2003). In contrast, neither *pmr2* (*mlo2*) plants nor *pen2 pmr2* plants were compromised in preinvasion resistance in *A. thaliana–M. oryzae* interactions (Fig. 6 and data not shown). This suggests that PMR2 (MLO2) has no function in preinvasion resistance in *A. thaliana–M. oryzae* interactions. However, in *A. thaliana*–powdery mildew interactions, PMR2 (MLO2) controls resistance together with the phylogenetically closely related paralogs MLO6 and MLO12, which act in partial functional redundancy (Consonni et al. 2006). Thus, it remains possible that a single *pmr2* (*mlo2*) mutation might not be sufficient to compromise the preinvasion resistance to *M. oryzae* in *A. thaliana*.

PMR4 controls callose synthesis at cell wall appositions (papillae) that form beneath infection and wound sites and are believed to provide a physical barrier to fungal penetration (Jacobs et al. 2003; Nishimura et al. 2003). In *A. thaliana–M. oryzae* interactions, callose accumulation, including papillary callose and whole-cell callose, occurred in attacked epidermal cells (Fig. 7), indicating that epidermal cells detect and respond to entry attempts by the fungus in both preinvasion and postinvasion resistance. In *pen2 pmr4* plants, although callose accumulation in the epidermal cells, including papillary callose and whole-cell callose, was significantly reduced, they still showed pre- and postinvasion resistance against *M. oryzae* (Fig. 6), suggesting that PMR4-dependent callose accumulation does not contribute to resistance to *M. oryzae* in *A. thaliana.*

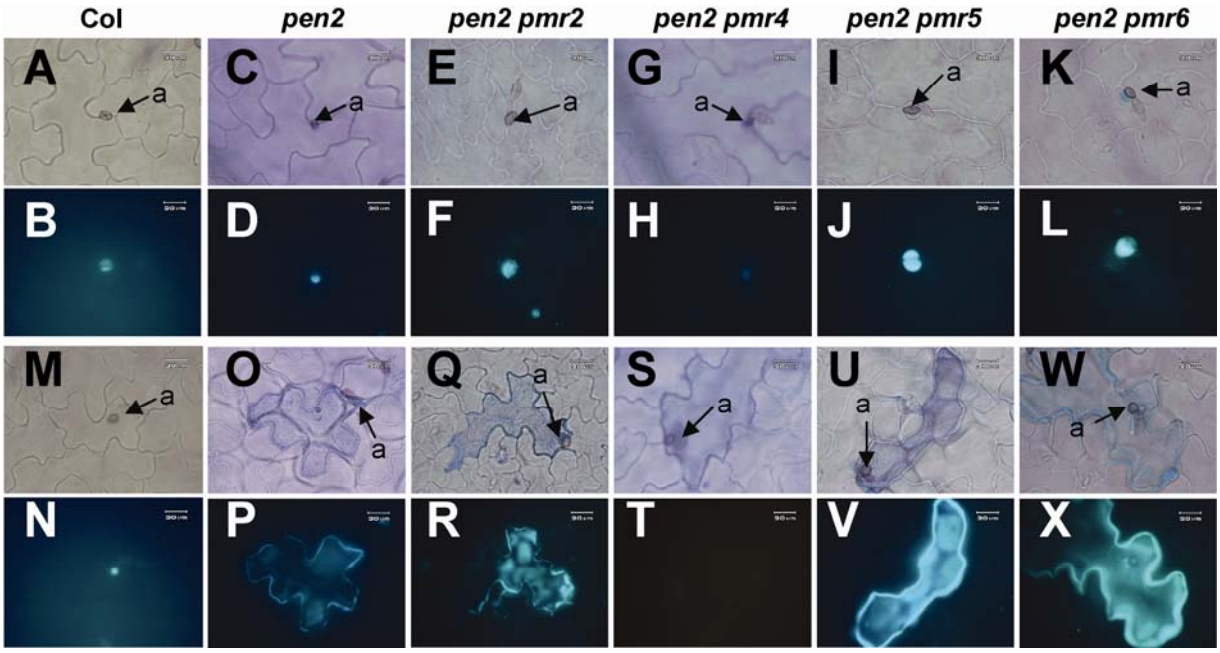


**Fig. 7.** Accumulation of callose at infection sites in double mutants. **A** through **L,** Papillary callose deposition at attempted penetration sites. Light-microscopic view of infection sites of **A,** Col-0; **C,** *pen2*; **E,** *pen2 pmr2* (*Atmlo2-7*); **G,** *pen2 pmr4*; **I,** *pen2 pmr5*; and **K,** *pen2 pmr6* plants at 3 days postinoculation (dpi). Papillary callose deposition at penetration sites of **B,** Col-0; **D,** *pen2*; **F,** *pen2 pmr2* (*Atmlo2-7*); **H,** *pen2 pmr4*; **J,** *pen2 pmr5*; and **L,** *pen2 pmr6* plants at 3 dpi were visualized by fluorescence microscopy following aniline blue staining. **M** through **X,** Callose deposition in whole epidermal cell. Light-microscopic view of infection sites of **M,** Col-0; **O,** *pen2*; **Q,** *pen2 pmr2* (*Atmlo2-7*); **S,** *pen2 pmr4*; **U,** *pen2 pmr5*; and **W,** *pen2 pmr6* plants at 3 dpi. Callose deposition at infection sites of **N,** Col-0; **P,** *pen2*; **R,** *pen2 pmr2* (*Atmlo2-7*); **T,** *pen2 pmr4*; **V,** *pen2 pmr5*; and **X,** *pen2 pmr6* plants at 3 dpi were visualized by fluorescence microscopy following aniline blue staining; a = appressorium. Bars = 20 μm.

*PMR5* encodes a member of a large family of plant-specific proteins of unknown function that is likely targeted to the endoplasmic reticulum and secretory pathway (Vogel et al. 2004). *PMR6* encodes a putative pectate lyase with proposed pectin-degrading activity localized at the cell wall (Vogel et al. 2002). Because the *pmr5 pmr6* double mutants show further increased resistance to powdery mildew compared with the respective single mutants, it seems likely that the two genes control parallel and independent defense responses (Vogel et al. 2004). Although *pen2 pmr5* plants showed significant increases in penetration ratio compared with *pen2* plants, *pen2 pmr6* plants did not show such an increase in *A. thaliana–M. oryzae* interactions (Fig. 6), suggesting that only PMR5 acts in preinvasion resistance to *M. oryzae* in a *pen2* background.

The heterotrimeric G protein is known to play a key role in stress responses as well as in development (Temple and Jones 2007). In *A. thaliana–M. oryzae* interactions, although *pen2 gpa1* plants tended to have a decreased penetration ratio compared with *pen2* plants, *pen2 agb1* plants had a significantly increased penetration ratio compared with *pen2* plants (Fig. 5). If G-protein-mediated signaling in *A. thaliana* follows the mechanism of action established in animal systems, loss of function of Gα could cause the release of free Gβγ, which leads to the activation of Gβγ signaling (Temple and Jones 2007). Our result supports this hypothesis and suggests that AGB1 is involved in the preinvasion resistance as a positive regulator and does not depend on the formation of a canonical Gαβγ heterotrimer.

We have shown that a block at the penetration step was the most prevalent form of resistance to *M. oryzae* in Col-0 and that the preinvasion resistance of Col-0 is stronger than that of L*er* (Fig. 1). To examine the genetic basis underlying the natural variation in the responses of *A. thaliana* to *M. oryzae,* we utilized a well-established set of RIL, derived from a Col × L*er* cross (Lister and Dean 1993). This analysis identified three QTL that govern resistance against *M. oryzae.* These QTL represented 39.0% of the total variation for this trait, suggesting that there are unidentified polymorphic loci controlling preinvasion resistance in *A. thaliana* (Fig. 2). We checked the identity of the genes in the identified QTL. The three QTL associated with this trait do not contain *PEN2*, *PMR5*, and *AGB1*, suggesting that a *PEN2*-independent pathway controls preinvasion resistance in *A. thaliana*. However, the QTL2 on chromosome 2 contained a GPA1 (*At2g26300*) coding sequence. In fact, the *pen2 gpa1* plants tended to have a decreased penetration ratio compared with *pen2* plants (Fig. 5), suggesting that GPA1 might function as a negative regulator in the preinvasion resistance. This supports the hypothesis that Gα sequesters Gβγ, which leads to the inactivation of Gβγ signaling. Although the amino acid sequences deduced from the *GPA1* genes is the same in Col-0 and L*er*, there might be a difference in levels of expression of GPA1 between Col-0 and L*er*, which might affect Gβγ signaling. QTL2 also contained an ERECTA (*At2g26330*) coding sequence. ERECTA has been identified as a positive mediator of resistance against necrotrophic infestation by both bacteria (*Ralstonia solanacearum*) (Godiard et al. 2003) and fungi (*Plectosphaerella cucumerina* and *Pythium irregulare*) (Llorente et al. 2005; Adie et al. 2007; Van Zanten et al. 2009). Interestingly, PEN2 is involved in the resistance in *A. thaliana–P. irregulare* interactions (Adie et al. 2007) and AGB1 is involved in the resistance in *A. thaliana–Plectosphaerella cucumerina* interactions (Llorente et al. 2005). Furthermore, analysis of *agb1 er* double mutants suggested that AGB1 may function in an ER developmental pathway regulating silique width (Lease et al. 2001). These results suggest that PEN2, AGB1, and ERECTA coordinately regulate the resistance to pathogens in *A. thaliana*. Further studies would be required to define more accurately the regions of the genome in which candidate genes are located. Identification of the polymorphisms underpinning these QTL would provide significant new insight into the expression of preinvasion resistance.

As shown in this study, *pen2* mutants allowed increased penetration of *M. oryzae*, which leads to an HR-like cell death (Fig. 3F). This indicates that *A. thaliana* NHR to *M. oryzae* is based upon two successive, multicomponent, and independently effective defense layers: PEN2-mediated preinvasion resistance and postinvasion resistance. It has been shown that PAD4 is involved in the postinvasion resistance to nonadapted powdery mildew in *A. thaliana* (Lipka et al. 2005). In the work reported here, the effects of *NahG, agb1,* and *pmr5* become visible only in a *pen2* genetic background, suggesting that *NahG, AGB1,* and *PMR5* might be involved in postinvasion resistance. However, none of the *pen2* double mutants, including *pen2 pad4*, supported growth of fungal colonies or sporulation (data not shown), indicating that postinvasion resistance is functional in these double mutants. Thus, PAD4 is not involved in postinvasion resistance in *A. thaliana–M. oryzae* interactions and *NahG, AGB1*, and *PMR5* contribute only to *PEN2*-mediated preinvasion resistance, indicating a role of unidentified genes in controlling postinvasion resistance to *M. oryzae* in *A. thaliana*. Alternatively, it could be that essential requirements that are needed to establish the infection are missing in *A. thaliana*.

Recently, Park and associates (2009) reported that some *M. oryzae* strains were able to infect *A. thaliana*. They also showed that the ability to produce melanized functional appressoria was not an absolute requirement for the infection of *A. thaliana* and that fungal metabolites were important determinants of the pathogenicity of *M. oryzae.* These data contrast with our studies. This discrepancy might be due to the difference of *M. oryzae* strains and experimental conditions used in the experiments. Future studies will be required to reveal the genetic and mechanistic requirements for NHR in *A. thaliana–M. oryzae* interactions. These studies may eventually be useful to improve resistance in rice.

## MATERIALS AND METHODS

### Plant material.

*A. thaliana* plants were grown under short-day conditions (9 h of light) at 22°C in a growth room. The following mutants and transgenic plants were used in this study: *pen1-1* (Collins et al. 2003); *pen2-1* (Lipka et al. 2005); *pen3* (*pdr8-1*) (Kobae et al. 2006); *Atmlo2-7* (SALK_079850); *npr1-1* (Cao et al. 1994); *sid2-1* (Nawrath and Metraux 1999); *pad1-1, pad2-1, pad3-1, pad4-1*, and *pad5-1* (Glazebrook and Ausubel 1994); *pmr1-1, prm2-1, pmr3-1, pmr4-1, pmr5-1*, and *pmr6-1* (Vogel and Somerville 2000); *eds1-2* (L*er*) (Aarts et al. 1998); *eds2-1, eds3-1, eds4-1, eds5-1, eds6-1, eds7-1, eds8-1, eds9-1, eds10-1, eds11-1, eds12-1, eds13-1, eds14-1, eds15-1*, and *eds16-1* (Glazebrook et al. 1996); *ein2-1* (Alonso et al. 1999); *coi1-1* (Xie et al. 1998); *jar1-1* (Staswick et al. 1992), *gpa1-4* and *agb1-2* (Chen et al. 2006); *rar1-21* (Tornero et al. 2002); *edm1-1* (Tor et al. 2002); *nho1-1* (Lu et al. 2001); *ndr1-1*(Century et al. 1995); and *NahG* (Lawton et al. 1995) (all Col-0 background except as noted). Among these, *pad4-1, eds5-1, sid2-1, NahG, coi1-1, jar1-1, ein2-1, pmr4-1, pmr5-1, pmr6-1*, and *Atmlo2-7*(*pmr2*) were used for intermutant crosses with *pen2-1*. Respective double homozygous F2 progeny were identified by polymerase chain reaction (PCR) using suitable cleaved amplified polymorphic sequence (CAPS) or derived CAPS markers; genotypes were verified in the subsequent (F3) generation. The *A. thaliana* accessions tested in this study were Col-0, L*er*, Ksk-1, No-0, and RLD-1.

For QTL analysis, the recombinant inbred (RI) population was obtained from the *Arabidopsis* Biological Resource Center. A subset of the RI populations (98 lines) was used for QTL analysis. Three plants for each RIL were grown and assayed for penetration resistance.

**Fungal material.**

The *M. oryzae* isolate Kita 1 (race 007) was incubated on oatmeal agar media in petri dishes at 25°C and the inoculum was prepared as described previously (Koga and Nakayachi 2004). For *M. oryzae* inoculations, 15-µl droplets ($10^4$ spores per milliliter) were applied to leaves of 4- to 5-week-old plants and plants were kept in conditions with saturating humidity until harvested.

**Cytology and quantification of fungal growth.**

For quantification of cell entry, the proportion of germinated fungal sporelings that developed appressorium was assessed on six leaves from six independent plants per experiment and genotype (minimum of 100 appressoria per leaf evaluated). Fungal penetration success on each genotype was quantified in at least three independent experiments. Plants were inoculated with *M. oryzae* and examined by fluorescence microscopy to monitor the autofluorescence resulting from the HR-like cell death triggered by penetration. For visualization of callose, samples were stained with aniline blue as described previously (Ishikawa et al. 2001). To assay for $H_2O_2$ accumulation, samples were stained with 3,3-diaminobenzidine tetrahydrochloride as described previously (Ishikawa et al. 2001). To visualize the epidermal cell death, the leaves were stained with lactophenol trypan blue and cleared with saturated chloral hydrate as described previously (Ishikawa et al. 2001).

**Treatment with catechol.**

To test for the effect of catechol on penetration resistance, 1 µM pyrocatechol or deionized water (mock) was applied to leaves 6 h prior to inoculation with *M. oryzae* as described previously (Consonni et al. 2006).

**QTL analysis.**

In all, 676 genetic markers were used for Col/L*er* RI populations as described previously (Singer et al. 2006). Using penetration ratio as a resistance index, composite interval mapping was carried out using QTL Cartographer, Model 6 (ver. 2.5) (Basten 1994). To reject false positives, a threshold of the LOD score for CIM analysis was calculated by a permutation test as described previously (Churchill and Doerge 1994) (1,000 times permutation at the significant level of $\alpha = 0.05$). The $R^2$ values were calculated by the program.

**Statistical analysis.**

Statistical analysis of data in this study is based on *t* test. Calculations were performed on a minimum of three data sets ($n = 3$), assuming two-sample equal variance and a two-tailed distribution. In the context of this report, we considered $P < 0.05$ to be a significant result.

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