Nonhost Resistance of *Arabidopsis thaliana* Against *Alternaria alternata* Involves both Pre- and Postinvasive Defenses but Is Collapsed by AAL-Toxin in the Absence of *LOH2*

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ABSTRACT

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The tomato pathotype of *Alternaria alternata* causes Alternaria stem canker on tomato depending upon the production of the host-specific AAL-toxin. Host defense mechanisms to *A. alternata*, however, are largely unknown. Here, we elucidate some of the mechanisms of nonhost resistance to *A. alternata* using *Arabidopsis* mutants. Wild-type *Arabidopsis* showed either no symptoms or a hypersensitive reaction (HR) when inoculated with both strains of AAL-toxin-producing and non-producing *A. alternata*. Yet, when these *Arabidopsis* penetration (*pen*)

mutants, *pen2* and *pen3*, were challenged with both strains of *A. alternata*, fungal penetration was possible. However, further fungal development and conidiation were limited on these *pen* mutants by postinvasion defense with HR-like cell death. Meanwhile, only AAL-toxin-producing *A. alternata* could invade lag one homologue (*loh*)2 mutants, which have a defect in the AAL-toxin resistance gene, subsequently allowing the fungus to complete its life cycle. Thus, the nonhost resistance of *Arabidopsis thaliana* to *A. alternata* consists of multilayered defense systems that include pre-invasion resistance via *PEN2* and *PEN3* and postinvasion resistance. However, our study also indicates that the pathogen is able to completely overcome the multilayered nonhost resistance if the plant is sensitive to the AAL-toxin, which is an effector of the toxin-dependent necrotrophic pathogen *A. alternata*.

Plants are continuously threatened by a large number of potentially pathogenic microbes that exhibit different lifestyles. Nevertheless, most plants are resistant to the majority of potential pathogens and susceptible to only a small number of adapted microbes. The term “nonhost resistance” is defined such that entire plant species must exhibit resistance to all genetic variants of a nonadapted pathogen species (25,27,29). Nonhost resistance is a consequence of multilayered defense mechanisms that include both constitutive barriers and inducible reactions (25,27,29). It is widely acknowledged that not only nonhost but also basal resistance mechanisms are elicited by recognition of non-self molecules, microbe-associated molecular patterns (MAMPs), or pathogen-associated molecular patterns (18,25,27,29). Certain successful pathogens that have evolved effectors can invade plants by interference of MAMP-triggered immunity, which is known as effector-triggered susceptibility. Subsequently, some plants enhance defense systems which are evoked by the recognition of effectors. This sequential co-evolution between plants and microbes and MAMP- or effector-triggered immunity and susceptibility forms the concept of plant immunity (18,27).

Several studies have been conducted on nonhost resistance. It is well known that *Arabidopsis* *PENETRATION* (*PEN*) mutants fail to defend against entry of nonadapted powdery mildews (6,22,33). *PEN1* encodes SYP121 (syntaxin of plants 121), a plasma membrane-resident syntaxin with a soluble N-ethylmaleimide-

sensitive factor attachment protein receptor (SNARE) domain (6). SNARE domain-containing proteins play a key role in vesicle-associated membrane fusion and secretion processes, including exocytosis and endocytosis. It is suggested that *PEN1* secretes cell wall precursors or antimicrobial compounds at the sites of attempted fungal invasion (3,21). *PEN2* encodes a peroxisome-associated glycosyl hydrolase, an atypical myrosinase that hydrolyzes 4-methoxynidol-3-ylmethylglucosinolates (4MI3G) as an in planta substrate for antifungal responses (4,22). *PEN3* encodes a plasma membrane-resident pleiotropic drug resistance ATP-binding cassette (ABC) transporter, PDR8 (33). *PEN3* is likely involved in exporting a toxic compound, whose synthesis is catalyzed by *PEN2*, at attempted fungal entry sites (3,21,33). One mechanism of nonhost resistance conferred by these three genes consists of two distinct secretory pathways, *PEN1* or co-operative *PEN2* and *PEN3* pathways (3,21). *PEN1* seems to be engaged exclusively for nonhost resistance against powdery mildews, whereas *PEN2* and *PEN3* contribute to resistance against these multiple parasites: *Colletotrichum gloeosporioides*, *Magnaporthe grisea*, *Phakopsora pachyrhizi*, *Pythium irregular*, and *Phytophthora infestans* (15,22–24,33).

Alternaria alternata (Fr.) Keissl. is a ubiquitous fungus that can be found on many kinds of plants and other substrata all over the world. While having a general potential for aggressiveness, most of them are unable to invade potential host plants due to nonhost or basal resistance (28,30). However, some of them are necrotrophic pathogens that produce host-specific toxins (HSTs) that confer fungal pathogenicity or virulence, leading to necrotic cell death only on susceptible plants. Specifically, HSTs are considered to be primary determinants of pathogenicity (in other words, effectors) that contribute to the establishment of susceptibility by suppression of plant defense responses (10,28,30).

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The tomato pathotype of *A. alternata*, the causal agent of Alternaria stem canker, produces host-specific AAL-toxin (12,37). AAL-toxin causes necrotic cell death on susceptible tomato plants and induces pathogen infection. It has been reported that the target of AAL-toxin is the enzyme sphinganine N-acyltransferase, which contributes to ceramide biosynthesis (13,32). The insensitivity of tomato to AAL-toxin is conferred by the resistance gene Alternaria stem canker gene 1 (*Asc-1*), a homolog of the yeast longevity assurance gene *LAG1* that encodes ceramide synthase, which appears to salvage the endoplasmic reticulum-to-Golgi transportation of glycosylphosphatidylinositol-anchored proteins by the production of an alternative ceramide (5). It has been shown that *Arabidopsis thaliana* *LAG1* homologues (lag one homologue [*LOH*]1, -2, and 3) are ceramide synthases and contribute to sensitivity or insensitivity to AAL-toxin (11,35).

Although the roles for HSTs as effectors which lead to disease susceptibility have been well characterized in some plant-pathogen interactions, there is little knowledge on the defense mechanisms in plants. In this study, we have analyzed the nonhost resistance against *Alternaria alternata* using *Arabidopsis* as a model system. Here, we demonstrate that *PEN2* and *PEN3* contribute to nonhost resistance against *A. alternata*. Microscopic analyses indicated that nonhost resistance in *Arabidopsis* to *A. alternata* consists of multilayered defense mechanisms that include pre-invasion resistance, including *PEN2* and *PEN3* and an unknown postinvasion resistance mechanism with a hypersensitive reaction (HR). Notably, the results suggest that the HST could entirely impair the multilayered nonhost resistance systems in the toxin-sensitive plants.

MATERIALS AND METHODS

Fungal strain and plant material. The tomato pathotype of *A. alternata* (synonym *A. alternata* f. sp. *lycopersici*, *A. arborescens*) strain As-27 which produces host-specific AAL-toxin, and nonpathogenic *A. alternata* strain O-94 which does not produce AAL-toxin, were maintained on potato dextrose agar (Difco Laboratories, Detroit) slopes or as 15% glycerol stocks at -80°C . The following *Arabidopsis* lines were used in this study: Columbia (Col-0), *pen1-1* (6), *pen2-1* (22), *pen2-2* (22), and *pen3-1* (33). The T-DNA insertion lines in *loh2-1* (SALK_018608) and *loh2-2* (SALK_024192) were obtained from the Arabidopsis Biological Resource Center (1). Plants were grown in sterilized soil in a growth chamber (Growth Cabinet MLR-350; Sanyo, Osaka, Japan) with a 10-h photoperiod at 22°C .

Inoculation. For pathogenicity tests, spores of the fungus were prepared as described previously (17). Droplets (5 μl) of spore suspensions (5×10^5 spores/ml) were placed onto the leaf surface. Inoculated plants were incubated in a moist chamber with a 10-h photoperiod at 22°C and lesion formation was observed at 7 days postinoculation. Infection behaviors were examined by microscopic observation, as described previously (7).

Microscopic observation. To detect generation of H_2O_2 in inoculated leaves, 3,3'-diaminobenzidine (DAB) staining was performed as described by Thordal-Christensen et al. (36). Inoculated leaves were collected after 7 days of inoculation and soaked in a DAB solution (1 mg/ml, pH 3.8) overnight. Subsequently, leaves were decolorized by ethanol. Ethanol was replaced with chloral hydrate solution (chloral hydrate, 8 g; glycerol, 1 ml; and H_2O , 2 ml) and leaves were observed under a light microscope (Nikon, Tokyo). The cell death on inoculated leaves was visualized by trypan blue staining. Inoculated leaves were boiled in a staining solution (lactophenol [lactic acid/glycerol/phenol/water, 1:1:1:1, by volume]/EtOH, 1:2 [vol/vol] and trypan blue at 2.5 mg/ml) for 1 min and incubated overnight at room temperature. Then, leaf samples were replaced in a chloral hydrate solution and observed with a light microscope. To visual-

ize callose deposition, inoculated leaves were fixed and decolorized in ethanol-acetic acid solution (3:1 [vol/vol]) until chlorophyll was removed. The leaves were then stained with aniline blue (0.1% [wt/vol]) in 100 mM K_2HPO_4 , pH 8.5 as described by Narusaka et al. (26). Callose deposition was examined under a fluorescent microscope (TE300-FN; Nikon). Fungal growth on inoculated leaves was visualized by histochemical staining using wheat germ agglutinin (WGA) Alexa Fluor 488 conjugate (Invitrogen, Carlsbad, CA). Inoculated leaves were harvested at 7 days postinoculation and soaked into WGA conjugate solution at 5 $\mu\text{g}/\text{ml}$ following the manufacturer's instructions. Fungal mycelium was observed under a fluorescent stereomicroscope (VB-G25; Keyence, Osaka, Japan).

Bioassay of AAL-toxin. Excised leaves were treated with different concentrations of the toxin solutions (0.01, 0.1, 0.5, and 1 $\mu\text{g}/\text{ml}$) by absorption through petioles. Leaves were incubated at 25°C for 4 days under fluorescent light.

Assay for antifungal component from leaves. Drops (30 μl) of spore suspensions (10^6 spores/ml) of O-94 were placed on leaves and incubated for 24 h. The suspensions on the leaves were harvested and centrifuged at $16,000 \times g$ for 5 min to remove germination spores. Spores of O-94 were suspended in the supernatant (5×10^5 spores/ml) and placed on a cellulose membrane (seamless cellulose tubing; Wako Pure Chemicals, Osaka, Japan). After 24 h, spore germination, appressorium formation, and infection hypha formation were observed under a light microscope.

Measurement of conidiation. Ten inoculated leaves were harvested at 10 days postinoculation and shaken vigorously in 1 ml of distilled water containing 0.01% (vol/vol) Tween 20. Samples were centrifuged (5 min) and, then, 700 μl of supernatant was discarded. Reproduced-spores were counted by using a hemocytometer.

Fungal biomass determination. Total genomic DNA was prepared from 10 inoculated leaves. Real-time polymerase chain reaction (PCR) was performed as described previously (9) and the primer pairs corresponding to *MAT1-1* idiomorph 3' flanking region of *A. alternata* (2) were used.

Reverse-transcription PCR. To determine expression of *LOH2* in *loh2* mutants, total RNA were extracted from plants with the Total RNA Extraction Kit Mini (Plant) (RBC Bioscience, Taipei, Taiwan) following the manufacturer's instructions. Total RNA was treated with DNase I (Takara, Ohtsu, Japan) to remove traces of contaminating DNA and 1 μg of RNA was converted into cDNA by the PrimeScript II 1st strand cDNA Synthesis Kit (Takara) according to the manufacturer's conditions. The primer pairs (AtLAG2RT-F, 5'-GTGTATGGTGTGGCTGCGTTG-3' and AtLAG2RT-R, 5'-CATCATCATCCTCTGAATCGG-3') corresponding to *LOH2* were used. As a control for equal cDNA amounts in each reaction, PCR was performed with primers for *elongation factor 1 α* (*EF1 α*) (AtEF-1RT-F, 5'-GATGATTTGCTGTTGTAACAAGATG-3' and AtEF-1RT-R 5'-ATACCA CCAATCTTGTAGACATCC-3'). Reverse-transcription (RT)-PCR was performed with the following conditions; initial step of 5 min at 95°C ; followed by 28 cycles for *LOH2* or 24 cycles for *EF1 α* of 15 s at 95°C , 15 s at 55°C , and 30 s at 72°C ; and a final step of 5 min at 72°C .

Statistical analysis. Analyses of variance (ANOVAs) for all fungal developments were performed by a one-way ANOVA on Excel and Tukey's test at a significance level of $P < 0.05$. Antifungal effect of elucidates from inoculated leaves on spore germination, appressorium formation, and infection hyphae formation were compared by two-way ANOVA ($P < 0.05$) on Excel.

RESULTS

Nonhost resistance to *Alternaria* infection was compromised in *pen* mutants. None of the known *A. alternata* strains infect

wild-type *Arabidopsis* plants and the plant is considered to be a nonhost to *A. alternata* (26). To assess the components of nonhost resistance to *A. alternata*, *Arabidopsis pen* mutants known to be defective in penetration resistance to various parasites were inoculated with the tomato pathotype of *A. alternata* strain As-27, whose pathogenicity depends on AAL-toxin. Wild-type Col-0 exhibited resistance to As-27 infection regardless of toxin production (Fig. 1A). Microscopic observation revealed that germination tubes were detected on the leaf surface of Col-0 (Supplemental Figure 1) and neither infection hyphae nor an HR concomitant with cell death was detected on Col-0 (Fig. 1A and B). On the other hand, macroscopic lesion formation developed on *pen2-1*, *pen2-2*, and *pen3-1* mutants inoculated with As-27, and microscopic observation revealed that infection hyphae were found (Fig. 1A and B), whereas *pen1-1* mutants did not develop any symptoms (Fig. 1A). All *pen* mutants were resistant to AAL-toxin and comparable with Col-0 at an AAL-toxin concentration of 1 $\mu\text{g/ml}$ (Fig. 1C). The same results in fungal infection were observed on *pen* mutants which were inoculated with nonpathogenic *A. alternata* strain O-94, which does not produce AAL-toxin.

Identification of a highly susceptible *Arabidopsis* mutant against *Alternaria* infection. AAL-toxin enables the tomato pathotype of *A. alternata* to infect susceptible plants with respect to *LAG1* homologue deficiency. Previously, Gechev et al. (11) reported that the *LAG1* homologue knockout mutant *Arabidopsis LOH2* from the Wassilewskija ecotype showed AAL-toxin sensitivity but susceptibility to fungal infection has not investi-

gated. To be able to compare the *LOH2* mutants alongside the *pen* mutants that were derived from Col-0, we searched for *LOH2* Col-0-derived mutants in public T-DNA insertion line collections (1). Two T-DNA insertion lines were obtained. Each line, SALK_018608 and SALK_024192, carrying an insertion in the third exon or the first intron, respectively, of *LOH2*, was named *loh2-1* and *loh2-2* (Fig. 2A). RT-PCR amplification to check for the *LOH2* transcript in *loh2-1* and *loh2-2* could not detect any gene expression in both mutant lines (Fig. 2B). An AAL-toxin sensitivity test revealed that both mutant lines exhibited high sensitivity to AAL-toxin (Fig. 2C). The wild-type *Arabidopsis* Col-0 was resistant to AAL-toxin and did not show any symptoms at a concentration of 1 $\mu\text{g/ml}$ (Fig. 2C). On the other hand, chlorotic cell death on both *loh2-1* and *loh2-2* mutants appeared at a low AAL-toxin concentration (0.1 $\mu\text{g/ml}$) and severity of cell death was dose dependent (Fig. 2C). The level of sensitivity to AAL-toxin in these mutants was comparable with the susceptible (*asc/asc*) tomato genotype (9,37,38). In addition to toxin sensitivity, both *loh2* mutants allowed fungal infection. Inoculation with As-27 to *loh2* mutants showed severe disease symptoms accompanied by necrotic cell death, and infection hyphae were observed (Fig. 2D and E). Meanwhile, nonpathogenic *A. alternata* strain O-94 failed to invade *loh* mutants.

Fungal biomass and conidiation. To assess fungal progression, fungal penetration, biomass, and conidiation were determined. Although there were no differences in spore germination among Col-0 and *Arabidopsis* mutants, infection hyphae formation differed. Both strains of *A. alternata* failed to penetrate

Fig. 1. *Alternaria alternata* infection on penetration (*pen*) mutants. *Arabidopsis* leaves were inoculated with the tomato pathotype of *A. alternata* strain As-27. **A**, Symptoms on the inoculated leaves. Droplets (5 μl) of spore suspensions (5×10^5 spores/ml) were placed onto the leaf surface. Symptoms were observed 7 days postinoculation. **B**, Microscopic observation of infection behavior of *A. alternata* on leaves. Triangles indicate infection hyphae. Scale bars = 100 μm . **C**, Sensitivity test of *pen* mutants to AAL-toxin. Excised leaves were treated with AAL-toxin solutions (1 $\mu\text{g/ml}$) by absorption through petioles. Symptoms were observed after 4 days.

Col-0 and rates of infection hyphae formation on *pen1-1* mutants were comparable with Col-0 (Fig. 3; Supplemental Figure 2). On the other hand, fungal infection hyphae formation increased to $\approx 10\%$ on *pen2-1*, *pen2-2*, and *pen3-1* mutants and there were no differences in the rate of infection hyphae formation of both strains among *pen2* and *pen3* mutants (Fig. 3). Numerous infection hyphae were detected and ≈ 30 and 48% of As-27 succeeded in penetration into *loh2-1* and *loh2-2* mutants, respectively (Fig. 3). Following penetration, mycelial growth was detected by the Alexa Fluor 488 conjugate of WGA, which exhibits green

fluorescence when binding to *N*-acetylglucosaminyl residues of fungal cell wall component. Despite spore germination of As-27 observed on Col-0, green fluorescence was not detected because fungal growth and expansion were not sufficient for fluorescence staining (Fig. 4A). On the other hand, remarkable fluorescence was observed in the combination of As-27 and *loh* mutants, and fungal mycelium expanded on the leaf (Fig. 4A). Although the level of toxin sensitivity of both *loh2* mutants was similar, the level of susceptibility to the pathogen was not the same. These results indicate that the truncated form of Asc-1 might have a

Fig. 2. Analysis of T-DNA-tagged lag one homologue (*LOH2*) disruptants. **A**, Location of T-DNA insertion in two T-DNA-tagged *loh2* disruptants, *loh2-1* and *loh2-2*. **B**, Reverse-transcription polymerase chain reaction (RT-PCR) analysis of *LOH2* expression in two *loh2* disruptants. Total RNA was obtained from leaves and 1 μg of RNA was used for cDNA synthesis. RT-PCR was performed with 28 and 24 amplification cycles for *LOH2* and *EF1α* respectively. Expression of *EF1α* was used for monitoring as a control for equal cDNA amounts in the reaction. **C**, Sensitivity test of *Arabidopsis* to AAL-toxin. Excised leaves were treated with different concentrations of toxin solutions (0.01, 0.1, 0.5, and 1 $\mu\text{g}/\text{ml}$) by absorption through petioles. Symptoms were observed after 4 days. **D**, *Alternaria alternata* infection on *loh2* mutants. Droplets (5 μl) of spore suspensions (5×10^5 spores/ml) of As-27 were placed onto the leaf surface. Symptoms were observed 7 days postinoculation. **E**, Microscopic observation of infection hyphae (triangles) of *A. alternata* on leaves. Scale bars = 100 μm .

dominant negative-like effect. In contrast, fungal penetration was detected on *pen2-1*, *pen2-2*, and *pen3-1* mutants but green fluorescence was not detected because fungal growth and expansion were not sufficient for fluorescence staining, the same as the result on Col-0 (Fig. 4A). No green fluorescence was observed on Col-0 and mutants inoculated with O-94. The amount of fungal DNA in inoculated leaves was determined by real-time PCR using *A. alternata* mating-type gene-specific primers. Both *loh2* mutants showed that the fungal biomass of As-27 in inoculated leaves was greater than other combinations (Fig. 4B). In spite of infection hyphae formation, fungal biomass levels on *pen* mutants were at the same level as on Col-0 (Fig. 4B). There were no differences in fungal biomass among Col-0 and mutants inoculated with O-94. In addition to fungal expansion, conidiation that occurs in a compatible interaction at the final stage of fungal infection was estimated. There were no differences among *pen* mutants and Col-0 inoculated with both strain of *A. alternata* (Fig. 4C), whereas significant conidiation was observed on *loh2-1* and *loh2-2* mutants inoculated with As-27 (Fig. 4C). The *loh2* mutation led to full infection and enabled completion of the life cycle of the As-27, and their interaction is regarded as a compatible interaction. These result indicated that *pen* mutants were compromised only in pre-invasion resistance and that postinvasion resistance against fungal development was still active.

HR-like cell death and H₂O₂ accumulation. There are two types of nonhost resistance: one of them is associated with HR cell death and the other shows no visible symptoms (25). According to Narusaka et al. (26), nonhost resistance of *Arabidopsis* to *A. alternata* was not accompanied by HR-like cell death. Likewise, trypan blue staining revealed that cell death was not detected on Col-0 inoculated with As-27 (Fig. 5A). DAB staining was performed to detect the generation of H₂O₂ that was associated with HR. H₂O₂ generation was not observed on Col-0 (Fig. 5B). Similarly, neither cell death nor H₂O₂ were detected in the *pen1-1* mutant inoculated with As-27 (Fig. 5A and B). In contrast, inoculation with As-27 on the *pen2-1*, *pen2-2*, and *pen3-1* mutants induced an HR-like cell death concomitant with H₂O₂ generation (Fig. 5A and B). When both *loh2* mutants were challenged with As-27, HR-like cell death and H₂O₂ accumulation were extensively detected (Fig. 5A and B). HR-cell death and H₂O₂ accumulation were observed on only *pen2* and *pen3* mutants inoculated with O-94 (Supplemental Figure 3).

Callose deposition. There are many reports on the role of formation of cell wall apposition or papillae in nonhost resistance (14); therefore, callose deposition, which is a major component of cell wall apposition, was evaluated. It has been reported that callose deposition was observed in Col-0 inoculated with *A. alternata* less than with *A. brassicicola* (26). Similarly, we detected slight callose deposition on Col-0 inoculated with As-27 (Fig. 5C). The degree of callose deposition on *pen1-1* mutants was comparable with Col-0 (Fig. 5C). By contrast, the number and size of cells with callose deposition were enlarged on *pen2-1*, *pen2-2*, and *pen3-1* mutants (Fig. 5C), similar to *A. brassicicola*-challenged Col-0 (26). Enormous callose deposition was detected on *loh2* mutants by inoculation with As-27 (Fig. 5C). Moderate callose deposition was observed on *pen2* and *pen3* mutants but not on *loh* mutants inoculated with O-94.

Antifungal activity of exudates from inoculated leaves. Previously, the role of ABC transporters as pumps for the release of antifungal compounds has been revealed in *Nicotiana* spp. (16). PEN3 is assumed to release antimicrobial compounds at the site of attempted fungal invasion (3,22). Therefore, we assessed the induction of antifungal compounds from inoculated leaves. Spores of O-94 were suspended in exudates which were collected from inoculated leaf surfaces, and spore germination, appressorium formation, and infection hyphae formation was assessed by microscopic observation. No differences in spore germination

and appressorium formation among the exudates from Col-0 and *pen* mutants were detected (Fig. 6). Furthermore, rate of infection hyphae formation was also similar among all plants (Fig. 6). These results suggest that antimicrobial compounds might not be released to the surface on *Arabidopsis* leaves during *A. alternata* infection.

DISCUSSION

The nonhost resistance of *Arabidopsis* to *A. alternata* has been described by Narusaka et al. (26). Likewise, we observed a nonhost resistance reaction to *A. alternata* infection in wild-type *Arabidopsis* Col-0, and this was represented as a failure to form lesions, fungal penetration, fungal progression, and conidiation. There are two types of nonhost resistance: one of them is associated with HR cell death and the other type produces no visible symptoms (25). Neither cell death nor infection hyphae were detected in the interaction between *A. alternata* and Col-0. In the infection process of *A. alternata*, formation of the infection hypha is the most critical step in initial establishment of successful invasion (7,28,30). We obtained the *LOH2* knockout mutants *loh2-1* and *loh2-2* in Col-0 and showed not only AAL-toxin sensitivity but also pathogen susceptibility. The sensitivity and susceptibility levels of both *loh2* mutants were similar to the susceptible host tomato genotype (9,37,38). Furthermore, the tomato pathotype of *A. alternata* markedly proliferated on both *loh2* mutants leaves and conidiation was found at a later stage of infection. The *loh2* mutants allowed the tomato pathotype of *A. alternata* not only to penetrate but also to complete its life cycle. In contrast, successful penetration was not observed in the combination of non-toxin-producing strain O-94/*loh2* mutants or toxin-producing strain As-27/wild-type Col-0. It was reported that induced resistance in host tomato plants by nonpathogenic O-94 was defeated by AAL-toxin treatment (7,38). The critical role of HSTs was considered to suppress the induction of general resistance specifically in susceptible plant (30). These results indicate that the interaction between *loh2* mutants and the tomato pathotype of *A. alternata* leads to full compatibility as a consequence of toxin sensitivity of *Arabidopsis* and AAL-toxin productivity of the pathogen.

Arabidopsis pen mutants have been well studied and shown to display a reduction in nonhost resistance not only to nonadapted

Fig. 3. Penetration ability of the tomato pathotype of *Alternaria alternata* strain As-27 on *Arabidopsis*. Microscopic observation of rate of infection hyphae formation on inoculated leaves at 7 days postinoculation. Data represent the mean of five independent experiments and error bars. Means with the same letter are not significantly different according to Tukey's test ($P < 0.05$).

powdery mildew but also to several parasites (15,22–24,33). In *Arabidopsis*, PEN1 seems to be engaged exclusively in nonhost resistance against powdery mildews, whereas PEN2 and PEN3 contribute to multiple nonhost pathogen resistance (15,22–24,33).

Similarly, our results show that only *pen2* and *pen3* mutants but not the *pen1* mutant resulted in infection of *A. alternata*. Although many studies have identified a large number of candidate defense genes involved in the processes of nonhost, basal, or

Fig. 4. Disease severity in *Arabidopsis* mutants inoculated with *Alternaria alternata* strain As-27. **A**, Fungal growth on the leaves. Fungal mycelia were stained with wheat germ agglutinin Alexa Fluor 488 conjugate. The blight red color of chlorophyll autofluorescence shows healthy leaf tissue and green color shows fungal mycelia. **B**, Comparison of fungal growth in *Arabidopsis* leaves inoculated with As-27 at 7 days postinoculation. Fungal biomass in the infected plants was estimated by quantifying fungal DNAs with real-time polymerase chain reaction using specific primers for the *MAT1-1* gene. The result shows relative values to inoculated Col-0 leaves for three independent experiments and error bars. An asterisk indicated significant difference according to Tukey's test ($P < 0.05$). **C**, Comparison of conidiation of *A. alternata* strain As-27 on inoculated leaves at 10 days postinoculation. Reproducing spores were harvested from 10 inoculated leaves and determined by using a hemocytometer. Data represent the mean of eight independent experiments and error bars. An asterisk indicated significant difference according to Tukey's test ($P < 0.05$).

Fig. 5. Hypersensitive reaction events on *Arabidopsis* mutants. *Arabidopsis* leaves were inoculated with *Alternaria alternata* strain As-27 and microscopic observation was conducted at 7 days postinoculation. **A**, Visualization of cell death by trypan blue staining. **B**, Detection of H₂O₂ generation by 3,3'-diaminobenzidine staining. **C**, Detection of callose deposition by fluorescence microscopy following aniline blue staining. Scale bars = 100 μ m.

general resistant to *A. alternata*, which consists of sequential multilayered defense mechanisms, the components of resistance have not been previously identified (7,8,28). In fact, expression of the *PEN3* gene was increased after *A. alternata* infection (26). This is the first report to identify a component of nonhost resistance to *A. alternata*. Meanwhile, the rate of *A. alternata* penetration on compatible *loh2* mutants was higher than on *pen* mutants, suggesting that the presence of other defense mechanisms for fungal penetration are still active against *A. alternata* in these *pen* mutants.

The roles of *PEN2* and *PEN3* are known, with *PEN2* described as an atypical myrosinase that hydrolyzes 4MI3G derived from tryptophan, and *PEN3* determined to be an ABC transporter that functions to discharge the *PEN2* product into the apoplastic space at the site of attempted fungal invasion (4,22). In addition to *PEN3*, an ABC transporter of *Nicotiana* spp. has been demonstrated to have a role as a pump for the release of antifungal compounds (16). We have evaluated the presence of toxic compounds in nonhost resistance of *Arabidopsis* against *A. alternata* but no antifungal activity was detected. In addition to the *PEN2* products, camalexin, an *Arabidopsis* phytoalexin, which is also derived from tryptophan, has been well characterized for its role for defense against necrotrophic pathogens *A. brassicicola* and *Botrytis cinerea* (20). However, because nonhost resistance in camalexin-null mutant *pad3-1* was not altered by *A. alternata* infection (26), it is suggested that camalexin is not involved in *Arabidopsis* penetration resistance to *A. alternata*. The infection-inhibiting factor which represses only infection hypha formation of *A. alternata* without antifungal effect has been identified from Japanese pear leaves (19). Involvement of such infection-inhibiting factors in nonhost resistance of *Arabidopsis* against infection with *A. alternata* needs to be further investigated.

Some studies have demonstrated that nonadapted pathogens succeeded in penetrating into *Arabidopsis pen* mutant cells but the full life cycle of the pathogen was not completed owing to effective postinvasion resistance associated with HR (22,24,33). Similarly, penetration of *A. alternata* on *pen2* and *pen3* mutants was concomitant with an HR-like cell death, H_2O_2 generation, and callose deposition following fungal development, such that the fungus was unable to complete its life cycle. In addition, H_2O_2 generation and callose deposition were extensively detected on *Arabidopsis* infected with the incompatible pathogen *A. brassicicola*; however, they were rarely observed by nonhost pathogen *A. alternata* infection (26). These results indicate that H_2O_2 generation and callose deposition was elicited by fungal penetration or, in other words, by the breakdown of pre-invasion resistance. Thus, HR and related events are considered to be part of postinvasion resistance to *A. alternata*. On the other hand, there have been several studies carried out using cytological analysis of host and nonhost plants during *A. alternata* infection (26,31,34). These studies have revealed that H_2O_2 generation and callose deposition were more distinct on the susceptible Japanese pear leaves than resistant leaves after pathogen penetration concomitant with cell wall and plasma membrane damage (31,34). Moreover, there was a report that H_2O_2 generation and cell death were induced in the *loh2* mutant by AAL-toxin treatment (11). The cell death in AAL-toxin-sensitive host plants induced by the toxin is a programmed cell death similar to the HR-type cell death in incompatible interactions (13). Those results suggested that "HR-like" cell death could be induced in both incompatible and compatible interactions even though the outcome of the cell death events is completely different pathologically. Further study will be necessary to fully understand the pathological roles of the cell death events induced in resistant *pen* and susceptible *loh2* mutants.

The HR symptoms observed on the *pen* mutants during *A. alternata* infection were similar to those observed on the in-

compatible Col-0 during *A. brassicicola* infection (26). Therefore, we can surmise that the nonhost interaction between *Arabidopsis* and *A. alternata* may advance in an incompatible interaction if there is a defect in *PEN* gene functions. In the interaction between *Arabidopsis* and *A. brassicicola*, it is well known that the jasmonic acid (JA) signaling pathway is involved in postinvasion resistance. Previously, we have reported that salicylic acid (SA) and JA signaling pathways are not important for induced resistance (in particular, pre-invasion resistance) in host plants to *A. alternata* (7,8). However, there were some reports that SA or JA signaling mutants with *pen* mutation differed from *pen* single mutants in responses to fungal postinvasion (23,33). Therefore, for future research, it should be very interesting to follow up postinvasion resistant mechanisms and explore the role of plant hormonal signaling in resistance to *A. alternata*.

In summary, we demonstrated that defense activation against *A. alternata* consists of multiple defense mechanisms that use pre-invasion resistance which involves *PEN2* and *PEN3* genes, as well as an unknown postinvasion resistance mechanism. In the nonhost interaction, *A. alternata* may only have to overcome one or two *PEN* genes to penetrate *Arabidopsis* leaves. However, postinvasion resistances which include an HR response effectively inhibit further fungal development. The finding suggests that *A. alternata* must overcome sequential multilayered defense mechanisms to establish compatible interactions on host plants. However, if such nonhost plants become sensitive to AAL-toxin, the toxin-producing pathogen can establish fully compatible interactions with the plant via the toxin-dependent interference of the resistance.

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Fig. 6. Antifungal activity test of exudates from leaf surfaces. Exudates were obtained after 24 h from leaf surfaces challenged by distilled water (DW) (dotted bar) or *Alternaria alternata* (black bar). Spores of *A. alternata* suspended in exudates and spore germination (GS), appressorium formation (A), and infection hyphae formation (IH) on cellulose membrane were determined after 24 h. Data represent the mean of four independent experiments. There were no significant differences among plants according to two-way analysis of variance ($P < 0.05$).

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