Barley MLO Modulates Actin-Dependent and Actin-Independent Antifungal Defense Pathways at the Cell Periphery*\[W]\[OA]

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Cell polarization is a crucial process during plant development, as well as in plant-microbe interactions, and is frequently associated with extensive cytoskeletal rearrangements. In interactions of plants with inappropriate fungal pathogens (so-called non-host interactions), the actin cytoskeleton is thought to contribute to the establishment of effective barriers at the cell periphery against fungal ingress. Here, we impeded actin cytoskeleton function in various types of disease resistance using pharmacological inhibitors and genetic interference via ectopic expression of an actin-depolymerizing factor-encoding gene, ADF. We demonstrate that barley (Hordeum vulgare) epidermal cells require actin cytoskeleton function for basal defense to the appropriate powdery mildew pathogen Blumeria graminis f. sp. hordei and for mlo-mediated resistance at the cell wall, but not for several tested race-specific immune responses. Analysis of non-host resistance to two tested inappropriate powdery mildews, Erysiphe pisi and B. graminis f. sp. tritici, revealed the existence of actin-dependent and actin-independent resistance pathways acting at the cell periphery. These pathways act synergistically and appear to be under negative control by the plasma membrane-resident MLO protein.

Plant cells attacked by fungal parasites respond by rapid cellular rearrangements and molecular reprogramming that lead to host cell polarization toward the potential intruder prior to invasion (Schmelzer, 2002; Lipka and Panstruga, 2005). These changes, generally assumed to contribute to a first line of defense, involve reorganization of the actin cytoskeleton, translocation of the cytoplasm and the nucleus, as well as focal deposition of cell wall material at incipient fungal entry sites (Schmelzer, 2002; Takemoto et al., 2003; Takemoto and Hardham, 2004). It is thought that the actin cytoskeleton plays a pivotal role for part of this cell polarization process by providing tracks for organelle and vesicle traffic. Consistent with this idea, pharmacological perturbation of the actin cytoskeleton has been reported to increase the incidence of fungal entry into attacked plant cells in a range of plant species that are not a natural host for these parasites (so-called non-host plants; Kobayashi et al., 1997a, 1997b; Yun et al., 2003; Shimada et al., 2006). It appears thus conceivable that pathogens that evolved means to establish compatible host-parasite interaction with a particular plant species may interfere with presumptive actin cytoskeleton-dependent defense processes. This may, in analogy to bacterial pathogens of mammals, involve the secretion of effector molecules that either directly or indirectly impinge on host actin cytoskeleton function. Whereas experimental evidence for this hypothesis is currently lacking from plant-fungus interactions, there is precedence from plant-bacteria interactions, suggesting that secreted microbial effector molecules may interfere with actin-dependent processes inside the host cell. Delivery of the bacterial type III effector AvrPto appears to be required for suppression of callose deposition in leaves of Arabidopsis (Arabidopsis thaliana) challenged with the bacterial pathogen, Pseudomonas syringae (Hauck et al., 2003). Focal callose deposition at attempted pathogen entry sites has been previously shown to be an actin-dependent process (Kobayashi and Hakuno, 2003).

Actin-binding proteins of the actin-depolymerizing factor (ADF)/cofilin family regulate, in concert with profilin and other actin-binding proteins, intracellular actin filament dynamics (Carlier et al., 1997; Theriot, 1997). ADFs are believed to increase the turnover (treadmilling) of filamentous actin (F-actin) by accelerating the rate-limiting depolymerization of globular monomeric actin (G-actin) from the pointed end of F-actin. ADFs are found...
in all eukaryotes examined to date (Maciver and Hussey, 2002). In plants, ADFs are encoded by medium-sized gene families with preferential expression of some genes in roots and pollen. Distinct plant ADF family members have been implicated in a range of biological processes, such as, for example, pollen tube elongation (Chen et al., 2002), flowering time, trichome development, cell expansion (Dong et al., 2001), cold acclimation (Ouellet et al., 2001), as well as gravity-oriented polarized rhizoid growth (Braun et al., 2004).

Barley (Hordeum vulgare) HvMlo and Arabidopsis AtMLO2 encode members of a family of plant-specific integral membrane proteins with seven membrane-spanning domains (Büschgés et al., 1997; Devoto et al., 1999, 2003). HvMLO and AtMLO2 are potentially targeted for pathogenesis by family members of the Erysiphales, common ascomycete pathogens that represent the causal agents of the powdery mildew disease in plants (Panstruga and Schulze-Lefert, 2003; Panstruga, 2005). Lack of HvMlo in homoyzogous barley mlo mutants results in broad-spectrum resistance that is effective against all known isolates of the barley powdery mildew fungus Blumeria graminis f. sp. hordei (Bgh). Barley mlo resistance is characterized by failure of Bgh sporelings to successfully enter epidermal host cells. Likewise, loss of AtMLO2 conditions resistance against multiple powdery mildew species that are generally able to successfully colonize Arabidopsis, such as Golovinomyces (formerly Erysiphe) cichoracearum and Golovinomyces orontii (Consonni et al., 2006). HvMLO and AtMLO2 each interact with the Ca$^{2+}$-sensor calmodulin (Kim et al., 2002; Bhat et al., 2005) and appear to inhibit vesicle-associated and SNARE protein-dependent defense reactions to powdery mildew fungi at the cell periphery (Collins et al., 2003; Schulze-Lefert, 2004; Panstruga, 2005).

Here, we used pharmacological as well as genetic means to interfere with the host actin cytoskeleton in barley-powdery mildew interactions. This enabled us to study the requirement of actin cytoskeleton function for basal resistance that attenuates fungal growth in compatible interactions. We also show a critical role for actin cytoskeleton function in mlo-mediated and non-host resistance that is highly effective against appropriate and inappropriate powdery mildew species, respectively. In sum, our findings suggest that the barley MLO protein modulates actin-dependent and actin-independent defense pathways at the cell periphery.

RESULTS

Pharmacological Interference with Actin Cytoskeleton Function Compromises Basal and mlo Resistance

Cytochalasins represent a compound class of well-characterized specific actin polymerization inhibitors (Cooper, 1987). Treatment of barley coleoptiles as well as leaf tissue of barley, wheat (Triticum aestivum), cucumber (Cucumis sativus), tobacco (Nicotiana spp.), and Arabidopsis with cytochalasins compromises resistance to pathogen entry in interactions with a range of inappropriate powdery mildew fungi and other biotrophic and hemibiotrophic fungal pathogens (Kobayashi et al., 1997a, 1997b; Yun et al., 2003). Application of the drug enabled fungal entry and haustorium differentiation in the non-host cells in a dose-dependent manner, suggesting that actin filaments play a crucial role in restricting cell access by non-host pathogens in higher plant species. To assess the role of actin filaments in basal defense and mlo-mediated resistance in the barley-powdery mildew interaction, we treated detached leaf sections of barley lines ‘Ingrid’ (Mlo genotype, susceptible) and the near-isogenic backcross (BC) line BC ‘Ingrid’ mlo-5 (resistant) with a range of cytochalasin E concentrations prior to spore inoculation with the host powdery mildew pathogen Bgh. In susceptible Mlo leaves, control treatments (0.25% dimethyl sulfoxide [DMSO]; v/v) resulted in approximately 60% of interaction sites in successful fungal entry and this incidence increased in the presence of cytochalasin E in a dose-dependent manner, reaching approximately 95% at a concentration of 1 μg/mL (supersusceptibility; Fig. 1A). Higher concentrations decreased the admittance rate to less than 10% at 25 μg/mL cytochalasin E, likely due to interference with the fungal actin cytoskeleton at elevated drug levels (Fig. 1A). Interestingly, highly efficient mlo resistance also exhibited dose-dependent sensitivity to cytochalasin E treatment. At a concentration of 1 μg/mL, we observed a fungal entry rate of approximately 55% compared to 0% in DMSO controls (Fig. 1B). Higher cytochalasin E concentrations decreased this incidence, which is consistent with fungitoxic activity at elevated drug levels (Fig. 1B). Collectively, these data demonstrate that pharmacological interference with the host actin cytoskeleton not only compromises non-host resistance, but also basal resistance to an appropriate pathogen as well as mlo resistance.

Genetic Interference with Actin Cytoskeleton Function as a Novel Tool to Study Actin-Dependent Biological Processes

In a previous study, Chen and coworkers reported that transient overexpression of a tobacco ADF (NtADF1) in pollen resulted in significant dose-dependent reduction of thin axial actin filaments and concomitant aggregation of filamentous actin in thick cables in the respective pollen tubes (Chen et al., 2002). This coincided with reduced tube growth of transformed pollen grains, which is reminiscent of pharmacological interference with pollen tube elongation by actin polymerization inhibitors (Chen et al., 2002). To examine whether ectopic ADF expression also affects the actin cytoskeleton in other plant cells, we introduced cDNA encoding an ADF family member that is known to be expressed in barley leaf epidermis (HoADF3;
see “Materials and Methods”) into the Gateway-based expression vector pUbi-Gate and ballistically transferred the resulting plasmid into barley leaf epidermal cells. Staining of actin filaments with the actin-specific fluorescent dye Alexa-Fluor phalloidin 488 revealed an intact actin filament network in control cells transformed with a plasmid encoding the reporter fluorophore dsRED (Fig. 2A; Supplemental Video S1). In contrast, phalloidin-stainable actin filaments were largely absent in leaf epidermal cells that were cotransformed with plasmids encoding dsRED and HvADF3, indicating that HvADF3 activity interferes with the integrity of the plant actin cytoskeleton (Fig. 2B; Supplemental Video S2). To assess whether the disappearance of phalloidin-stainable actin filaments upon HvADF3 expression coincides with compromised actin cytoskeleton function, we investigated peroxisome motility as an indicator of actin-dependent transport processes. Unlike in animals, where peroxisomes shuttle on microtubules, plant peroxisomes move exclusively along actin filaments and pharmacological interference with the actin cytoskeleton leads to rapid global arrest of peroxisome movement and assembly of the organelles in globular aggregates (Jedd

Figure 1. Pharmacological interference with the host actin cytoskeleton compromises basal and mlo resistance in barley. Barley leaf sections of either the Mlo (‘Ingrid’; A) or mlo (BC ‘Ingrid’ mlo-5; B) genotype were vacuum infiltrated with various concentrations of the actin polymerization inhibitor cytochalasin E as described in “Materials and Methods.” Subsequently, leaves were inoculated with Bgh conidiospores and successful entry in epidermal cells evaluated by microscopy at 48 h postinoculation. Data shown represent mean ± so from one (of three) representative experiment in which four leaves on 100 interaction sites each were evaluated. Asterisks beside columns indicate P ≤ 0.05 (Student’s t test) compared to the negative control (0 μg/mL cytochalasin E).

Figure 2. Ectopic HvADF3 expression compromises actin cytoskeleton integrity and function in barley epidermal cells. A and B, Barley leaf sections of the Mlo (‘Golden Promise’) genotype were ballistically transformed with either a dsRED reporter construct only (A) or a dsRED reporter construct plus an effector construct encoding HvADF3 (B). Subsequently, leaf sections were stained with Alexa-Fluor phalloidin 488 as described in “Materials and Methods” at 48 h after particle bombardment. Micrographs were taken with a confocal laser-scanning microscope and each represents a two-dimensional projection of a series of optical sections. Both micrographs show representative epidermal cells of the respective transformations. AF, Alexa-Fluor-stained actin filaments. C and D, Barley leaf sections of the Mlo (‘Golden Promise’) genotype were ballistically transformed with either a GFP-PTS1 peroxisome marker construct only (C) or a GFP-PTS1 marker construct plus an effector construct encoding HvADF3 (D). Subsequently, leaves were inspected by epifluorescence microscopy at 60 h after particle bombardment. Both micrographs show a representative epidermal cell of the respective transformations. N, Nucleus; P, motile peroxisomes; PA, immobile peroxisome aggregates.
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Indirect Evidence for N-Terminal Phosphorylation of HvADF3

Previous studies revealed reversible phosphorylation of vertebrate/plant ADFs at a conserved N-terminal and Chua, 2002; Mano et al., 2002; Mathur et al., 2002). We expressed a GFP variant with a C-terminal peroxisomal targeting sequence (GFP-PTS1; see “Materials and Methods”) with or without HvADF3 in barley leaf epidermal cells and studied peroxisomal movement by epifluorescence microscopy at 60 h after particle bombardment. Whereas in >95% of the epidermal cells peroxisomes steadily traveled with the typical saltatory movements when transformed with pUbGFP-PTS1 alone (Fig. 2C; Supplemental Video S3), we observed significant reduction in peroxisome velocity (in approximately 20% of transformed cells) or total arrest of organelle movement (in approximately 55% of transformed cells) upon HvADF3 coexpression. The latter was frequently associated with the formation of peroxisome aggregates (Fig. 2D; Supplemental Video S4), which is consistent with previously reported effects on organelle motility by pharmacological actin cytoskeleton interference (Mathur et al., 2002). Despite this drastic perturbation of organelle motility and actin cytoskeleton architecture, affected cells remained alive because individual peroxisomes still exhibited few saltatory movements (Supplemental Video S4). Collectively, these data indicate that ectopic HvADF3 expression effectively impedes both actin cytoskeleton integrity and function in barley epidermal cells. We conclude that genetic interference with the actin cytoskeleton by transient ectopic expression of HvADF3 represents an experimental alternative to pharmacological perturbation of actin filaments and propose ADF overexpression as a novel tool to study actin-dependent biological processes.

Genetic Interference with Actin Cytoskeleton Function in Plant-Microbe Interactions

To examine the effectiveness of genetic interference with actin cytoskeleton function in the context of the barley-powdery mildew interaction, we ectopically expressed HvADF3 in barley leaf epidermal cells of both Mlo and mlo genotypes. We observed enhanced host cell entry (supersusceptibility, Mlo genotype) and partial break down of mlo resistance, respectively (Fig. 3, A and B), which is reminiscent of the results obtained upon pharmacological actin cytoskeleton disturbance (see above; Fig. 1). To assess whether this consequence of ectopic ADF expression is specific for particular ADF isoforms, we tested the effect of transient expression of various heterologous Arabidopsis ADFs (AtADF1, AtADF2, AtADF3, AtADF4, AtADF5, AtADF6, AtADF7, AtADF9, and AtADF12) on mlo-mediated powdery mildew resistance. We found that most Arabidopsis ADFs were able to confer enhanced fungal entry (Fig. 3C). However, the incidence of host cell invasion varied considerably among Arabidopsis ADF family members. To distinguish whether the failure of AtADF3, AtADF4, and AtADF9 to mediate enhanced host cell entry was due to paralog-specific functional differences or ADF protein instability in the heterologous system, we generated translational fusions of these isoforms to the C terminus of the yellow fluorescent protein (YFP) coding region. When transiently expressed in single barley leaf epidermal cells, two of the three fusion proteins exhibited both typical YFP-specific fluorescence (as revealed by epifluorescence microscopy; data not shown), as well as elevated incidence of fungal entry in mlo genotypes. These findings suggest that the respective unfused, native Arabidopsis ADFs might be unstable upon heterologous expression in barley leaf epidermis, whereas they are stabilized by translational fusion to YFP (data not shown). Taken together, these findings indicate that genetic interference with actin cytoskeleton function is not restricted to a particular ADF isoform, but seems to be a general feature upon ectopic ADF expression.

Figure 3. Genetic interference with the host actin cytoskeleton compromises basal and mlo resistance in barley. Barley leaf sections of either the Mlo ('Golden Promise'; A) or mlo (BC 'Ingrid' mlo-3; B and C) genotype were ballistically transformed with either a GUS reporter construct only (GUS) or a GUS reporter construct plus an effector construct (here: encoding various ADF variants). Subsequently, leaves were inoculated with a high density of Bgh conidiospores, stained for GUS activity, and microscopically evaluated as described in “Materials and Methods.” Data shown represent mean ± SD from at least three experiments in which, as a minimum, 100 GUS-stained cells each were evaluated. Asterisks beside columns indicate *P < 0.05 (Student’s t test) compared to the negative control (GUS only).
Ser residue (Ser-3 in vertebrate ADFs and Ser-6 in maize [Zea mays] ADF3) as a regulatory posttranslational modification (Agnew et al., 1995; Moriyama et al., 1996; Smertenko et al., 1998). The phosphorylated ADF forms exhibit dramatically reduced G- or F-actin binding and are inactive in in vitro polymerization/depolymerization assays. Single amino acid replacements of Ser-6 in maize ADF3 resulted in mutant proteins that either mimic constitutive phosphorylation (S6D) or generate a nonphosphorylatable variant (S6A; Smertenko et al., 1998). We introduced analogous amino acid replacements in HvADF3 and tested the resulting variants in the transient single-cell gene expression assay. In Mlo wild-type plants, the HvADF S6A variant conferred higher host cell entry rates than the wildtype protein (85% versus 77%, respectively), whereas the S6D variant mediated the opposite effect (57% versus 77%, respectively; Fig. 3A). Likewise, the S6A and S6D variants altered elevated fungal entry mediated by wild-type HvADF3 in the mlo-3 mutant background (16%) in opposite directions (23% and 6%, respectively; Fig. 3B). These data are consistent with the previous notion that an N-terminal Ser residue of plant ADFs might be a phosphorylation target site and that phosphorylation of this residue reduces ADF activity in planta (Smertenko et al., 1998; Chen et al., 2002).

**Actin Cytoskeleton Function Is Dispensable for Barley Race-Specific Powdery Mildew Resistance**

Isolate-specific immunity is a common form of barley disease resistance against the Bgh pathogen (Jørgensen, 1994). This type of resistance requires the presence of a host-resident race-specific resistance (R) gene and a matching pathogen-encoded avirulence (Avr) gene. It is thought that resistance is triggered upon indirect or direct recognition of the AVR effector by a cognate R-protein (van der Biezen and Jones, 1998). To assess the contribution of actin cytoskeleton function in R gene-mediated resistance, we interfered pharmacologically (by application of cytochalasin E) and genetically (via ectopic expression of pharmacologically (by application of cytochalasin E) variants in the transient single-cell gene expression assay. We introduced analogous amino acid replacements in HvADF3 and tested the resulting variants in the transient single-cell gene expression assay. Previous pharmacological studies revealed a potent role for the actin cytoskeleton in non-host resistance of various plant-microbe interactions (Kobayashi et al., 1997a, 1997b; Yun et al., 2003; Shimada et al., 2006). To genetically test interference with actin cytoskeleton function in the context of inappropriate plant-fungus interactions, we inoculated HvADF3-transfected mlo and Mlo genotype leaves with conidiospores of either the wheat powdery mildew fungus B. graminis f. sp. tritici (Bgt) or the pea (Pisum sativum) powdery mildew fungus Erysiphe pisi, respectively. In the Mlo genotype, ectopic HvADF3 expression compromised resistance to fungal entry, to different degrees, against both tested inappropriate powder mildew species (Fig. 5, A–D). The much lower frequency of E. pisi invasion in comparison to Bgt might reflect the phylogenetic distance of the respective powdery mildew species (Erysiphe diverged from Blumeria approximately 100 million years ago; Mori et al., 2000). Surprisingly, this HvADF3-dependent inhibition of resistance to the inappropriate powdery mildews was not observed in the mlo genotype (Fig. 5, A and C). To validate that the differential infection phenotypes in Mlo and mlo genotypes was due to the presence or absence of Mlo, we coexpressed Mlo together with HvADF3 in the mlo genotype. This revealed that indeed presence or absence of Mlo dictates whether ectopically expressed HvADF3 is able to compromise forma specialis or non-host resistance (Fig. 5E). We infer an Mlo-dependent role for the actin cytoskeleton in the context of forma specialis and non-host resistance.

**Ectopic Expression of Bacterial Type III Effector Proteins Reveals AvrPto-Dependent Aberrant Callose Deposition in mlo Genotypes**

Several enteroinvasive bacterial human pathogens secrete so-called type III effector proteins that are required for pathogenesis. Some of these effectors have been shown to obstruct host actin cytoskeleton function. Well-known examples include the Cys protease.
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YopT (Yersinia spp.), the GTPase-activating protein ExoS (Pseudomonas aeruginosa), as well as the guanine nucleotide exchange factor SopE and the ADF/cofilin-competing protein SipA (both from Salmonella typhimurium; McGhie et al., 2004; for review, see Barbieri et al., 2002). Interference with the host actin cytoskeleton is thought to contribute to suppression of antimicrobial defense and may also stimulate the internalization of intracellularly acting microbes. Likewise, the secreted type III effector AvrPto of the plant pathogenic bacterium P. syringae pv tomato is believed to serve a role as a suppressor of a cell wall-associated defense response (Hauck et al., 2003).

To test whether bacterial effector proteins known to impede actin cytoskeleton function and/or suspected to act as defense suppressors may interfere with successful fungal host cell entry in the barley-powdery mildew interaction, we ectopically expressed YopT, ExoS, SopA, SipA, or AvrPto in single-leaf epidermal cells of either the susceptible Mlo or the resistant mlo genotype, respectively. Whereas none of the tested effectors derived from human pathogenic bacteria altered the infection phenotype upon Bgh challenge (data not shown), ectopic expression of AvrPto frequently resulted in an aberrant accumulation pattern of the (1–3)-β-D-polyglucan callose in the mlo, but not the Mlo, genotype (Fig. 6, A–D). Local paramural callose deposition is a widespread plant response upon various abiotic or biotic stress cues and is commonly seen in cell wall appositions (papillae) that are formed at the respective stress sites. Whereas callose deposition in the Mlo genotype was exclusively focal and restricted to incipient fungal entry sites (Fig. 6, A and B), additional scattered callose deposits were frequently found in the mlo genotype (Fig. 6, C and D). Despite their nonfocal appearance, the latter were usually also in spatial proximity to attempted pathogen entry sites. Collectively, this result corroborates the previous notion that AvrPto function is linked to pathogen-triggered focal callose deposition (Hauck et al., 2003). It further reveals a novel and unexpected link between AvrPto function, callose deposition, and MLO function.

**DISCUSSION**

Previous pharmacological studies using the actin-depolymerizing drug cytochalasin provided evidence for a contribution of host actin cytoskeleton function in non-host resistance at the cell wall to various inappropriate pathogens (Kobayashi et al., 1997a, 1997b; Yun et al., 2003; Shimada et al., 2006). Here, we present pharmacological as well as genetic evidence for the involvement of host actin cytoskeleton function in additional forms of disease resistance. These include basal resistance against a virulent mildew isolate in the Mlo (wild-type) genotype and mlo-mediated resistance (Figs. 1, 3, and 5). Because perturbation of actin-dependent processes partially compromised each of these three

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**Figure 4.** Pharmacological or genetic interference with the host actin cytoskeleton does not affect race-specific resistance in barley. A, Barley leaf sections of various genotypes (Mlo Mla1, line P01; Mlo Mla6, line P03; Mlo Mlg, line P21; or mlo-3, line P22) were pressure infiltrated with either the solvent DMSO (0.25% [v/v]), the actin polymerization inhibitor cytochalasin E (5 μg/mL), or the microtubule polymerization inhibitors propyzamide (15 μg/mL) or oryzalin (30 μg/mL) as described in “Materials and Methods.” Subsequently, leaves were inoculated with Bgh (either isolate K1 or A6) conidiospores and successful entry in epidermal cells evaluated by microscopy at 48 h postinoculation. Data shown represent the results of one set of experiments with the average and sd of three to four independent leaves per genotype and treatment. The asterisk above the column indicates P < 0.05 (Student’s t test) compared to the negative control (DMSO). The experiment was repeated once with similar results. B, Barley leaf sections of various genotypes (Mlo Mla1, Mlo Mla6, Mlo Mlg) were ballistically transformed with either a GUS reporter construct only (GUS) or a GUS reporter construct plus an effector construct encoding HvADF3. Subsequently, leaves were inoculated with a high density of Bgh (either isolate K1 or A6) conidiospores, stained for GUS activity, and microscopically evaluated as described in “Materials and Methods.” Data shown represent mean ± sd from at least three experiments in which, as a minimum, 100 GUS-stained cells each were evaluated.

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resistance types, manifested in each case by an increased incidence of pathogen entry, these immune responses appear to be mechanistically related. Indeed, a potential mechanistic and genetic overlap of barley host, non-host, and mlo-mediated powdery mildew resistance has been previously proposed (Peterhäuser et al., 1997; Collins et al., 2003; Trujillo et al., 2004; for review, see Humphry et al., 2006). However, an apparent complete obstruction of actin cytoskeleton function only partially compromised resistance against entry of powdery mildew fungi in an mlo genotype and in the tested non-host interactions (Figs. 1B, 3B, and 5, A and C), suggesting that other actin-independent mechanisms contribute to effective blockade of host cell entry that might become rate limiting in the absence of MLO. This notion is further corroborated by the fact that host cell entry rates were consistently higher in Mlo than in mlo genotypes upon either pharmacological or genetic interference with the host actin cytoskeleton (Figs. 1A, B, and E) or E. pisi (C and D) conidiospores, stained for GUS activity, and microscopically evaluated as described in “Materials and Methods.” A, C, and E, Quantitative assessment of powdery mildew cell entry rates. Data shown represent mean ± SD from at least three experiments in which, as a minimum, 100 GUS-stained cells each were evaluated. Asterisks beside columns indicate $P < 0.05$ (Student’s t test) compared to the negative control (GUS only). B and D, Micrographs of transformed, GUS-stained barley leaf epidermal cells successfully penetrated by sporelings of inappropriate powdery mildew species. CS, Conidiospore; EC, GUS-stained epidermal cell; ESH, elongating secondary hyphae; H, haustorium.

The existence of more than one resistance mechanism at the cell periphery might also help to explain the perplexing finding that a nonfunctional actin cytoskeleton permitted pathogen entry of the inappropriate E. pisi and Bgt powdery mildews only in the presence of Mlo, whereas this effect was seen in interactions with the host powdery mildew, Bgh, in both the presence or absence of Mlo (compare Fig. 3, A and B, with 5, A and C). We hypothesize that, in the absence of MLO, an actin-independent subset of defense responses at the cell periphery becomes rate limiting for the inappropriate powdery mildew species, E. pisi and Bgt. In contrast, for the host pathogen Bgh, actin-dependent defense responses are rate limiting in the mlo genotype (Fig. 3B). Thus, the inappropriate pathogens might need both presence of MLO (to suppress or bypass actin-independent defense responses) as well as a nonfunctional actin cytoskeleton for host cell entry. Unlike this, a compromised actin cytoskeleton function suffices to allow host cell entry by Bgh, implying that the compatible powdery mildew must have evolved means to corrupt or avoid actin-dependent...
and actin-independent resistance responses. Because cell entry rates in interactions with Bgh are cumulative upon interference with either resistance response (Figs. 1, A and B, and 3, A and B), the two pathways must act synergistically rather than sequentially. On the basis of this model, full resistance to Bgh in mlo null mutants can be best explained by suppression of both pathways via MLO (Fig. 7). Quantitative differences in the efficiency of resistance responses at the cell wall to host and inappropriate powdery mildew species may reflect molecular coadaptation of as yet uncharacterized fungal effectors for resistance suppression in their respective host plants (Panstruga, 2003).

Small monomeric G-proteins of the ROP (RAC of plants) class are well-known modulators of actin cytoskeleton organization in animal cells (Burridge and Wennerberg, 2004). A barley ROP isoform, HvRACB, was recently shown to contribute to focal actin reorganization upon Bgh attack and expression of a constitutive active HvRACB variant in single barley epidermal cells increased the incidence of fungal entry (supersusceptibility) in an MLO-dependent manner (Schultheiss et al., 2002, 2003; Opalski et al., 2005). Interestingly, in tobacco, a ROP isoform was shown to regulate the actin-binding and depolymerizing activity of a pollen-specific NtADF, thereby controlling pollen tube tip growth (Chen et al., 2003). Our data potentially link HvADF and HvRACB activity, as well as the actin cytoskeleton, to one process acting synergistically together with MLO-modulated actin-independent resistance at the cell periphery (Fig. 7). Unlike HvRACB, ectopic expression of the antiapoptotic Bax inhibitor gene HvBI1 increased the rate of Bgh entry in both Mlo and mlo barley genotypes (Hückelhoven et al., 2003). Ectopic HvBI1 expression also increased the entry rate in both Mlo and mlo genotypes in interactions with the inappropriate Bgt powdery mildew, whereas HvBI1 gene silencing did not alter fungal entry rates even in interactions with Bgh (Hückelhoven et al., 2003; Eichmann et al., 2004). Hence, HvBI1 cannot be clearly assigned to the actin-dependent or actin-independent resistance mechanism at the cell periphery, but rather may indirectly impinge on either process only upon HvBI1 overexpression.

In marked contrast to the resistance forms discussed above, both our pharmacological as well as genetic data suggest that a range of isolate-specific resistance specificities requires neither actin microfilaments nor microtubules (Fig. 4). This result corroborates previous genetic findings indicating that R gene-mediated resistance might mechanistically at least partially differ from basal defense in compatible interactions. For example, mutations in a number of functionally homologous barley and Arabidopsis genes required for R gene-mediated resistance (e.g. SGT1, RAR1, HSP90) do not affect basal resistance against virulent pathogens (Austin et al., 2002; Azevedo et al., 2002; Muskett et al., 2002; Takahashi et al., 2003; Hein et al., 2005). Vice versa, mutations in a subset of genes implicated in basal penetration resistance (e.g. barley Ror1 and Ror2; Freialdenhoven et al., 1996) do not interfere with isolate-specific resistance (Peterhansel et al., 1997; Trujillo et al., 2004). However, Arabidopsis EDS1 and PAD4 represent two genes, each of which is required for basal and isolate-specific resistance (mediated by R genes of the so-called TIR-NBS-LRR class) as well as for non-host immunity (Falk et al., 1999; Feys et al., 2001; Lipka et al., 2005). Likewise, tobacco NbHSP90, NbSGT1, and barley HvRom1 act in both R gene-mediated and non-host resistance (Peart et al., 2002; Kanzaki et al., 2003; Freialdenhoven et al., 2005). In conclusion, these genetic studies reveal evidence for the existence of both distinct and shared components in basal, isolate-specific, and non-host immunity.

How might actin cytoskeleton function contribute to plant defense? A wealth of studies revealed that plant cells undergo substantial cellular alterations upon fungal attack, leading to host cell polarization toward the site of attempted ingress (e.g. Kobayashi et al., 1994; Skalamera and Heath, 1998; Bhat et al., 2005; Opalski et al., 2005; for review, see Schmelzer, 2002; Lipka and Panstruga, 2005). This process includes major rearrangements of the cytoskeleton, translocation of the cytoplasm and the nucleus, as well as focal

Figure 6. Ectopic expression of the bacterial effector AvrPto results in aberrant pathogen-triggered callose deposition in leaf epidermal cells of the mlo geno-

type. barley leaf sections of either the Mlo (‘Golden Promise’; A and B) or mlo (BC ‘Ingrid’ mlo-3; C and D) genotype were ballistically transformed with a

GUS reporter construct plus a construct encoding AvrPto. Subsequently, leaves were inoculated with a high density of Bgh conidia, stained for GUS activity (at 48 h postinoculation) as well as for callose, and microscopically evaluated as described in “Materials and Methods.” Micrographs were either taken under UV excitation (A and C) or by applying mixed bright-field/UV illumination (B and D). Arrows indicate aniline blue-stained callose accumulation. ACD, aberrant callose deposition.
accumulation of HvMLO and AtPEN1/HvROR2 in a plasma membrane microdomain at prospective fungal entry sites. Similar polar reshuffling activities were also observed during mycorrhizal symbiosis and upon local mechanical stimulation of individual plant cells, suggesting that mechanoperception is sufficient to trigger cellular polarization (Russo and Bushnell, 1989; Gus-Mayer et al., 1998; Timonen and Peterson, 2002). Recent identification of plasma membrane-resident syntaxins (Arabidopsis AtPEN1 and barley HvROR2) required for non-host and mlo resistance in Arabidopsis and barley, respectively, indicates a potential role for vesicle-mediated exocytosis in antifungal defense (Collins et al., 2003; Schulze-Lefert, 2004). It is conceivable that cytoskeletal rearrangements following fungal attack form the basis for rapid and efficient organelle motility and productive focal exocytosis (DePina and Langford, 1999). Thus, cell polarization in reaction to fungal attack might be the result of an integrated response triggered by mechanosensors and immune receptors.

Although heterologous ectopic expression of the AvrPto effector of the plant-pathogenic bacterium, P. syringae, did not alter Bgh infection phenotypes in Mlo or mlo plants (data not shown), this resulted in aberrant callose deposition only in mlo genotypes (Fig. 6). In mlo mutants, multiple spontaneous callose deposits are frequently seen in single epidermal cells in a developmentally controlled manner, suggesting that the mutants are sensitized for the formation of aberrant cell wall appositions (Wolter et al., 1993). Ectopic expression of AvrPto in Arabidopsis compromised defense-associated callose deposition in the cell wall upon challenge with the bacterial pathogen P. syringae and permitted enhanced bacterial growth (Hauck et al., 2003). Unexpectedly, loss of callose accumulation at powdery mildew entry sites in Arabidopsis GLUCAN SYNTHASE-LIKE5/POWDERY MILDEW RESISTANT4 (GSL5/PMR4) mutants was recently shown to result in enhanced disease resistance rather than enhanced susceptibility (Jacobs et al., 2003; Nishimura et al., 2003). Unlike Arabidopsis GSL5/PMR4 mutants, barley Mlo and mlo cells ectopically expressing AvrPto retain the ability for callose accumulation beneath attempted fungal entry sites (Fig. 6), possibly accounting for the unaltered infection phenotypes. The additional irregular callose deposits seen only in the mlo mutant upon ectopic expression of AvrPto might reflect the combined effect of perturbing defense-associated callose deposition by the bacterial effector and sensitization for the formation of spontaneous cell wall appositions in the absence of MLO.

Whereas we demonstrated pharmacologically and genetically that the host actin cytoskeleton has an important role in terminating powdery mildew entry into plant cells, it is conceivable that actin filaments serve an alternative role at later stages during fungal pathogenesis. Once under control by the fungal intruder, continuous nutrient supply via the haustorium to the ephithetically growing hyphae is essential for the biotrophic lifestyle of powdery mildews. This phase might involve cytoskeleton-dependent transport processes toward haustorial complexes. Consistent with this, individual actin filaments in epidermal cells that were successfully colonized by a fungal powdery mildew sporeling are frequently directed toward the haustorium and even cover the tips and/or body of the fungal feeding organ (Opalski et al., 2005). The exact role of the actin cytoskeleton at these later stages of fungal pathogenesis remains an open question to date.

**MATERIALS AND METHODS**

**Plant and Fungal Material**

The following barley (Hordeum vulgare) lines were used for this study: ‘Ingrid’ (Mlo), ‘Golden Promise’ (Mlo), ‘110’ (near-isogenic line in ‘Ingrid’ background containing Mla12), ‘P01’ (near-isogenic line in ‘Pallas’ background containing Mla1), ‘P03’ (near-isogenic line in ‘Pallas’ background containing Mla6 and Mla14), ‘P21’ (near-isogenic line in ‘Pallas’ background containing Mlg), BC ‘Ingrid’ mlo-3, and BC ‘Ingrid’ mlo-5. All barley seedlings were grown at 20°C and 16 h light/8 h darkness in a protected environment. Bgh isolates K1 (AvrMla1, virMla6, virMla12, virMlg) and A6 (virMla1, AvrMla6, AvrMla12, AvrMlg) were propagated on barley lines ‘110’ and ‘P01’, respectively, for mutual exclusion. Bgt isolate JIW2 was propagated on an anonymous susceptible wheat (Triticum aestivum) cultivar. The anonymous pea (Pisum sativum) powdery mildew isolate was assigned as Eriosipte pisi by ribosomal spacer DNA analysis. The E. pisi strain was maintained on susceptible pea plants (‘Linga’).

**DNA Constructs**

Full-size coding sequences of HvADF3 (Unigene 2146 of barley HarvEST database, assembly 31, version 1.51; http://harvest.ucr.edu/) were PCR amplified based on sequence information obtained from EST clones of the epidemisper specific HO barley cDNA library (Zierold et al., 2005). This cDNA library was established using epidermal peels of powdery mildew-challenged primary leaves as a source. Likewise, full-size coding sequences of AtADF1 (At3g46010),
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AIAFD2 (At3g6000), AIAFD3 (At5g59880), AIAFD4 (At4g59880), AIAFD5 (At2g136700), AIAFD6 (At4g31200), AIAFD7 (At4g25980), AIAFD9 (At4g34970), and AIAFD12 (At4g52360) gene nomenclature according to Feng et al., 2006) were amplified by reverse transcription (RT)-PCR from RNA that was extracted from rosette leaves of Arabidopsis (Arabidopsis thaliana) ecotype Columbia (Col-0). Single amino acid substitutions S6A and S6D in HvADF3 were introduced by PCR mutagenesis. ADF coding sequences were integrated into suitable expression vectors (pUbI-nos or pUbI-Gate, in which expression is driven by the maize [Zea mays] polyubiquitin promoter) by either conventional restriction enzyme-based cloning or via Gateway recombination. The gene encoding a peroxisome-targeted GFP variant was generated via polytional restriction enzyme-based cloning or via Gateway recombination. The pDSK519, pGEX2T-Det al., 2002), were PCR amplified from plasmids pIM157, pcDNA3.1-SipA, previously described (Kim et al., 2002).

Pharmaceutical Treatments

For drug (cytochalasin E, oryzalin, propyzamide; Sigma-Aldrich) application, two different methods were employed: Either the lower (axial) epidermis of 2- to 5-cm sections of barley first leaves was removed, specimens were floated on a solution containing the respective compound (in 0.25% [v/v] DMSO), and vacuum applied (27 mm Hg, 10 min). Subsequently, the upper (abaxial) epidermis of the leaf sections was inoculated with Bgh conidiospores. Alternatively, the solutions were pressure infiltrated into barley leaves using a syringe lacking a needle. Forty-eight hours postinoculation leaves were cleared and epiphytic fungal structures stained with Coomassie Brilliant Blue for microscopic analysis.

Single-Cell Gene Expression

Ballistic transformation of detached barley leaves was carried out as previously described (Schweizer et al., 1999; Elliott et al., 2005). Bombarded specimens were inoculated with high densities of powdery mildew conidiospores and GUS staining performed 48 h postinoculation (Schweizer et al., 1999). Epiphytic fungal structures were marked by Coomassie Brilliant Blue. Leaf epidermal cells attacked by the appressorial germ tube of powdery mildew sporelings were microscopically evaluated for the presence or absence of haustoria. Penetration success was calculated as the number of transformed cells that exhibit one or multiple haustoria in relation to the total number of transformed cells attacked by powdery mildew sporelings.

Actin Filament and Callose Staining

Actin microfilaments were stained as described previously (Kobayashi et al., 1997a) with slight modifications (Opalski et al., 2005). Leaf segments (4 × 4 mm in size) were fixed in 3.7% formaldehyde in 1× PIPES buffer, pH 6.8, at room temperature for 1 h. After washing in 1× PIPES and 1× phosphate-buffered saline (PBS; pH 6.8), leaf segments were treated with 0.5% Triton X-100 in 1× PBS (pH 6.8) at room temperature for 1 h. The specimens were washed with 1× PBS (pH 6.8), then with 1× PBS (pH 7.4). Following three rinse cycles, leaf segments were stained with Alexa-Fluor 488 phalloidin (Molecular Probes; 0.66 μM in 1× PBS [pH 7.4]). To promote uptake of the dye, vacuum infiltration was performed three times for 20 s at 27 mm Hg. Subsequently, samples were stored at room temperature for 2 to 3 h in the dark. Finally, leaves were rinsed with 1× PBS (pH 7.4). Specimens were mounted in 1× PBS (pH 7.4) on glass slides and observed by confocal laser-scanning microscopy. Stacks of approximately 40 images were used for two-dimensional projections.

For callose staining, cleared leaves were rinsed in 50% ethanol, briefly washed in water, and then stained overnight in 150 mM KH₂PO₄ (pH 9.5) containing 0.01% aniline blue. For epifluorescence microscopy, samples were mounted in 50% glycerol and inspected by UV excitation.

Supplemental Data

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

Supplemental Video S1. Alexa-Fluor phalloidin staining in a dsRED expressing a barley epidermal cell.

Supplemental Video S2. Alexa-Fluor phalloidin staining in a barley epidermal cell coexpressing dsRED and HvADF3.

Supplemental Video S3. Expression of GFP-PTS1 in a barley leaf epidermal cell.

Supplemental Video S4. Coexpression of GFP-PTS1 and HvADF3 in a barley leaf epidermal cell.

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LITERATURE CITED


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