Barley MLO Modulates Actin-Dependent and Actin-Independent Antifungal Defense Pathways at the Cell Periphery[1][W][OA]

Marco Miklis, Chiara Consonni, Riyaz A. Bhat[2], Volker Lipka[3], Paul Schulze-Lefert, and Ralph Panstruga*

Max Planck Institute for Plant Breeding Research, D–50829 Cologne, Germany

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 actin filaments (Carlier et al., 1997). In addition, ADF/cofilins might nucleate the assembly of new actin filaments (Dos Remedios et al., 2003). ADFs are found

[1] This work was supported by the GABI non-host consortium, by the Max-Planck Society, and by the Deutsche Forschungsgemeinschaft (grant nos. PA861/1–1 and PA861/4–1 to R.P.).

[2] Present address: Commissariat à l'Energie Atomique, Cadarache, 13 108, St. Paul Lez Durance, France.

[3] Present address: Sainsbury Laboratory, John Innes Centre, Colney Lane, Norwich NR4 7UH, UK.

* Corresponding author; e-mail panstrug@mpiz-koeln.mpg.de; fax 49–221–5062–353.

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantphysiol.org) is: Ralph Panstruga (panstrug@mpiz-koeln.mpg.de).

[W] The online version of this article contains Web-only data.

[OA] Open Access articles can be viewed online without a subscription.

www.plantphysiol.org/cgi/doi/10.1104/pp.107.098897

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 (Büschges 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 homozygous 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 (*HvADF3*;

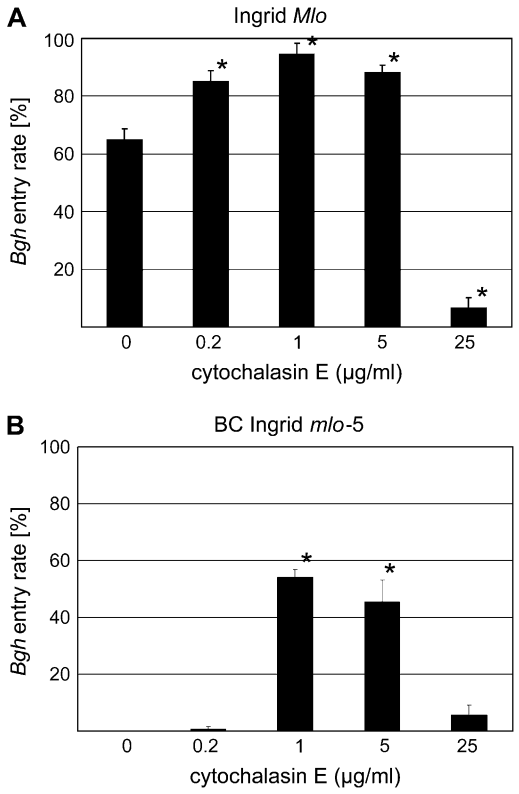

Figure 1. Pharmacological interference with the host actin cytoskeleton compromises basal and *mlo* resistance in barley. Barley leaf sections of either the *Mlo* ('Ingrid'; A) or *mlo* (BC 'Ingrid' *mlo*-5; B) genotype were vacuum infiltrated with various concentrations of the actin polymerization inhibitor 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 ± SD 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).

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

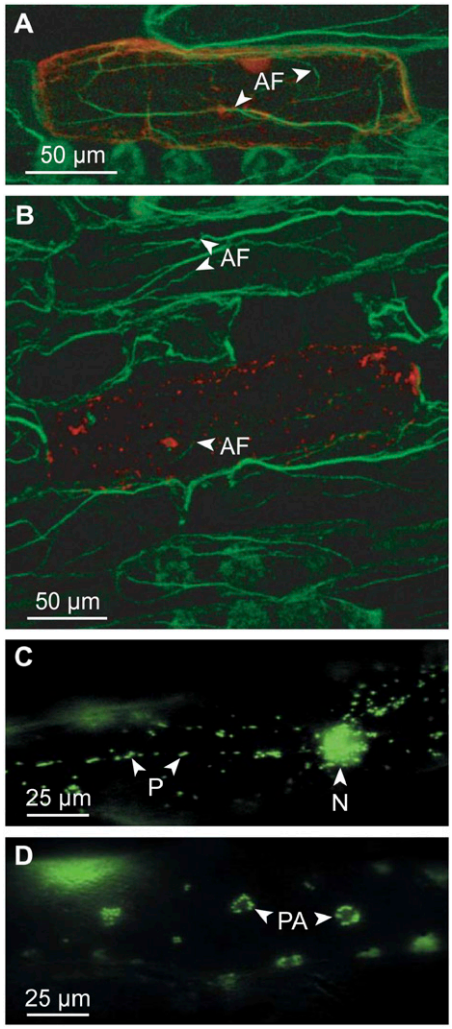

Figure 2. Ectopic *HvADF3* expression compromises actin cytoskeleton integrity and function in barley epidermal cells. A and B, Barley leaf sections of the *Mlo* ('Golden Promise') genotype were ballistically transformed with either a dsRED reporter construct only (A) or a dsRED reporter construct plus 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.

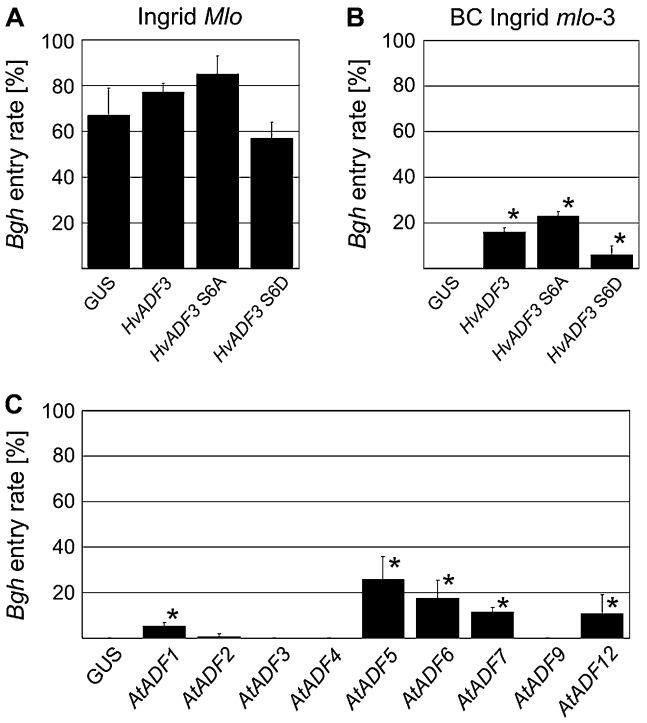

Figure 3. Genetic interference with the host actin cytoskeleton compromises basal and *mlo* resistance in barley. Barley leaf sections of either the *Mlo* ('Golden Promise'; A) or *mlo* (BC 'Ingrid' *mlo*-3; B and C) genotype were ballistically transformed with either a GUS reporter construct only (GUS) 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).

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 pUbi-GFP-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 nonfused, 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.

Indirect Evidence for N-Terminal Phosphorylation of HvADF3

Previous studies revealed reversible phosphorylation of vertebrate/plant ADFs at a conserved N-terminal

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 wild-type 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) as well as genetically (via ectopic expression of *HvADF3*) with the actin filament network in the context of three race-specific barley-powdery mildew interactions conditioned by *R* gene *Mla1*, *Mla6*, or *Mlg*. The activity of at least two of these *R* genes involves cell-autonomous function and can thus be monitored in single-leaf epidermal cells attacked by *Bgh* (Halterman et al., 2001; Zhou et al., 2001). All tested *R*/*Avr* gene combinations, namely, *Mla1*/*AvrMla1*, *Mla6*/*AvrMla6*, and *Mlg*/*AvrMlg*, were affected neither by pharmacological nor by genetic disturbance of actin cytoskeleton function (Fig. 4, A and B). These data suggest that race-specific resistance might be mechanistically distinct from non-host, basal, and *mlo*-mediated immunity that share a requirement for actin cytoskeleton function.

We also tested the effect of drugs known to impede microtubule polymerization (oryzalin and propyzamide, respectively; Morejohn et al., 1987; Akashi et al., 1988). Except for somewhat enhanced susceptibility in the *Mla1* genotype upon oryzalin treatment, application of these compounds did not change the incidence of *Bgh* entry in either *mlo* or isolate-specific resistance (*Mla1*/*AvrMla1*, *Mla6*/*AvrMla6*, *Mlg*/*AvrMlg*; Fig. 4). The result of oryzalin on *Mla1* resistance is likely not due to direct interference with microtubule function since propyzamide did not provoke this effect. However, we can not dismiss the possibility that oryzalin was more potent in disturbing microtubules than propyzamide under our conditions. In sum, the data of our pharmacological experiments suggest that microtubule functions are dispensable for all tested forms of disease resistance.

Mlo-Dependent Activity of the Actin Cytoskeleton in Forma Specialis and Non-Host Resistance

Previous pharmacological studies revealed a potential 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 powdery 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

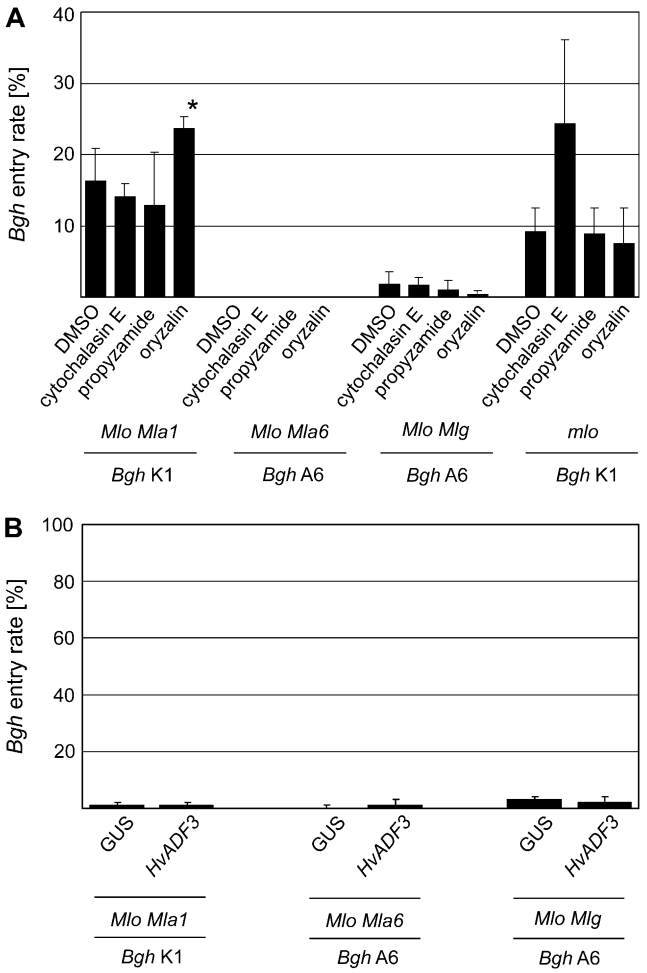

Figure 4. Pharmacological or genetic interference with the host actin cytoskeleton does not affect race-specific resistance in barley. A, Barley leaf sections of various genotypes (*Mlo Mla1*, line P01; *Mlo Mla6*, line P03; *Mlo Mlg*, line P21; or *mlo*-3, line P22) were pressure infiltrated with either the solvent DMSO (0.25% [v/v]), the actin polymerization inhibitor cytochalasin E (5 μg/mL), or the microtubule polymerization inhibitors propyzamide (15 μM) or oryzalin (30 μM) 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.

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

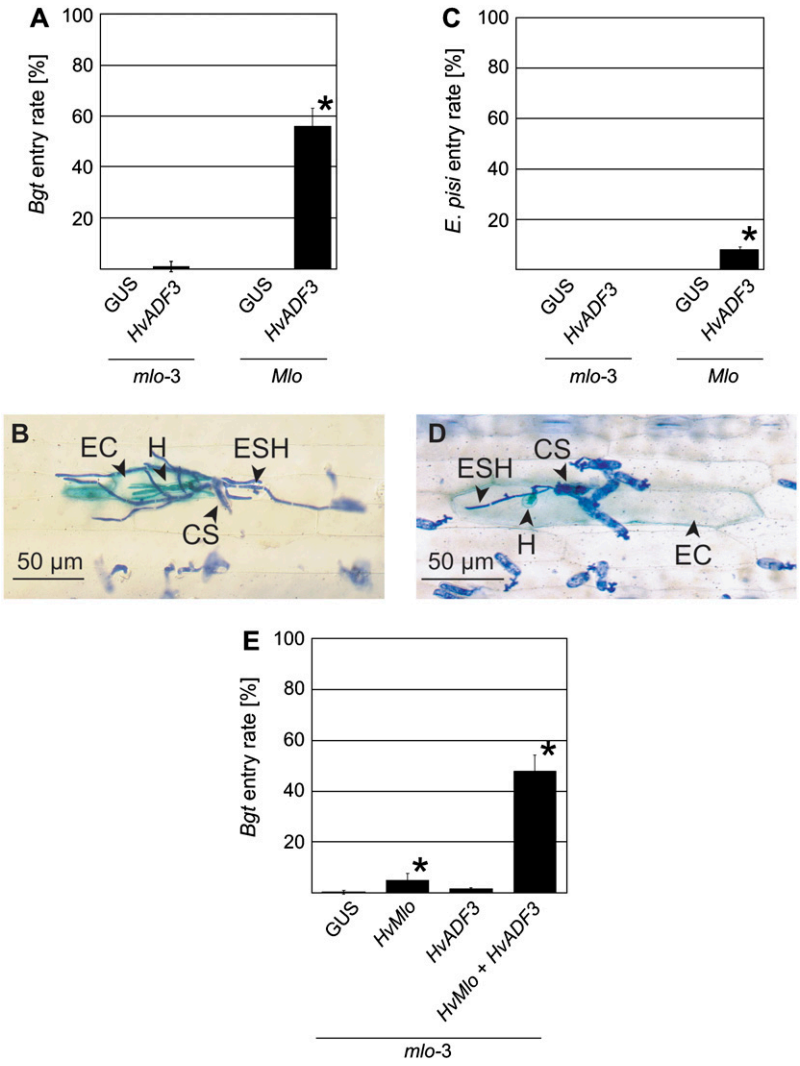

Figure 5. Genetic interference with the host. Actin cytoskeleton compromises non-host resistance in an *Mlo*-dependent manner. Barley leaf sections of either the *Mlo* ('Golden Promise') or *mlo* (BC 'Ingrid' *mlo*-3) genotype were ballistically transformed with either a GUS reporter construct only (GUS) or a GUS reporter construct plus one or two effector constructs (here: encoding HvADF or HvMLO). Subsequently, leaves were inoculated with a high density of either *Bgt* (A, 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.

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 (Peterhänsel 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 blockage 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. 1, A and B, and 3, A and B), implying that at least a component of MLO-dependent fungal entry occurs independently of the actin cytoskeleton.

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

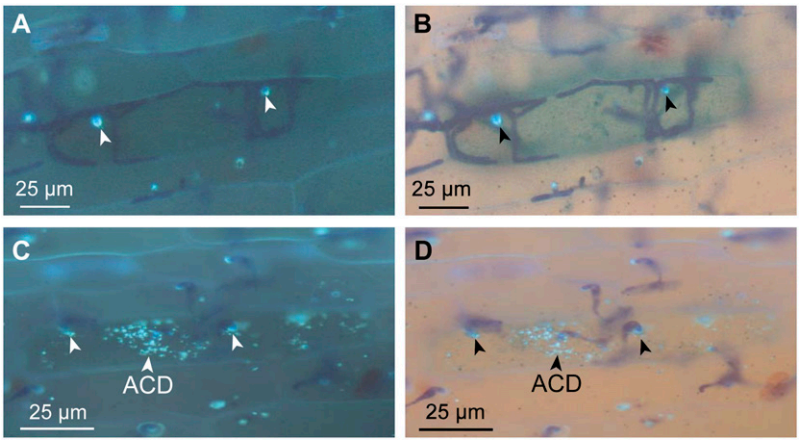

Figure 6. Ectopic expression of the bacterial effector *AvrPto* results in aberrant pathogen-triggered callose deposition in leaf epidermal cells of the *mlo* genotype. Barley leaf sections of either the *Mlo* ('Golden Promise'; A and B) or *mlo* (BC 'Ingrid' *mlo*-3; C and D) genotype were ballistically transformed with a GUS reporter construct plus a construct encoding AvrPto. Subsequently, leaves were inoculated with a high density of *Bgh* conidiospores, 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.

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 (Peterhänsel 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; Škalamera 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

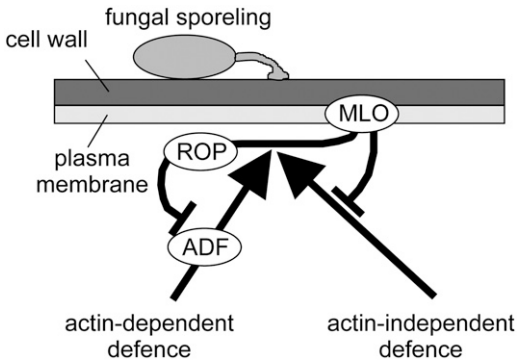

Figure 7. Model of MLO-modulated antifungal defense at the cell periphery. Actin-dependent and actin-independent defense response pathways contribute to limit fungal entry into barley wild-type (*Mlo* genotype) host epidermal cells. The plasma membrane-resident MLO protein acts as negative regulator of both defense pathways. The small monomeric G-protein ROP and the actin-modulating protein ADF operate in the actin-dependent branch. Based on the ability of constitutive ROP to confer enhanced host cell entry (supersusceptibility) in an MLO-dependent manner (Schultheiss et al., 2003), ROP is associated with the negative regulatory activity of MLO and may either directly or indirectly affect ADF activity (e.g. via regulation of its phosphorylation status [Chen et al., 2003]).

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 depositions 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 epiphytically 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*), 'I10' (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 'I10' 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 *Erysiphe 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 epidermis-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),

AtADF2 (At3g46000), *AtADF3* (At5g59880), *AtADF4* (At5g59890), *AtADF5* (At2g16700), *AtADF6* (At2g31200), *AtADF7* (At4g25590), *AtADF9* (At4g34970), and *AtADF12* (At5g52360; 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 polymerase PCR by adding the tripeptide motif Ser, Arg, Leu (SRL, a so-called PTS1-targeting sequence) to the C terminus of GFP (Jedd and Chua, 2002; Reumann, 2004). The *GFP-PTS1* sequence was introduced into expression vector pUbi-Gate by Gateway recombination. Plasmids pUbi-Mlo-nos, pUbi-GUS-nos (pUGN), and pUbi-dsRED-nos, employed for ectopic expression of barley *Mlo* or for marking transformed cells, respectively, were previously described (Kim et al., 2002).

cDNAs encoding the bacterial effectors YopT (Iriarte and Cornelis, 1998), SipA (McGhie et al., 2004), and AvrPto (Salmeron and Staskawicz, 1993), as well as catalytic domains of ExoS (Pederson et al., 2002) and SopE (Buchwald et al., 2002), were PCR amplified from plasmids pIM157, pcDNA3.1-SipA, pDSK519, pGEX2T-Δ96ExoS, and pGEX2T-Δ78SopE, respectively, using primer pairs that rendered PCR products suitable for Gateway cloning. Subsequently, amplicons were introduced into expression vector pUbi-Gate.

Pharmacological Treatments

For drug (cytochalasin E, oryzalin, propyzamide; Sigma-Aldrich) application, two different methods were employed: Either the lower (adaxial) 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 KH_2PO_4 (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.

ACKNOWLEDGMENTS

We thank Ralph Hückelhoven and Krystina Opalski for providing their phalloidin-based actin filament-staining protocol prior to publication, as well as Patrick Schweizer and Uwe Zierold for making available a range of barley cDNA clones. We also acknowledge Alfred Wittinghofer, Guy Cornelis, Vassilis Koronakis, and John Rathjen for providing plasmids containing the coding sequences of bacterial effectors ExoS, SopE, YopT, SipA, and AvrPto, respectively. We are grateful to Markus Frank for his continuous support.

Received March 15, 2007; accepted April 14, 2007; published April 20, 2007.

LITERATURE CITED

Agnew BJ, Minamide LS, Bamburg JR (1995) Reactivation of phosphorylated actin depolymerizing factor and identification of the regulatory site. J Biol Chem **270:** 17582–17587

Akashi T, Izumi K, Nagano E, Enomoto M, Mizuno K, Shibaoka H (1988) Effects of propyzamide on tobacco cell microtubules in vivo and in vitro. Plant Cell Physiol **29:** 1053–1062

Austin MJ, Muskett P, Kahn K, Feys BJ, Jones JDG, Parker JE (2002) Regulatory role of *SGT1* in early *R* gene-mediated plant defenses. Science **295:** 2077–2080

Azevedo C, Sadanandom A, Kitagawa K, Freialdenhoven A, Shirasu K, Schulze-Lefert P (2002) The RAR1 interactor SGT1, an essential component of *R* gene-triggered disease resistance. Science **295:** 2073–2076

Barbieri JT, Riese MJ, Aktories K (2002) Bacterial toxins that modify the actin cytoskeleton. Annu Rev Cell Dev Biol **18:** 315–344

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

Braun M, Hauslage J, Czogalla A, Limbach C (2004) Tip-localized actin polymerization and remodeling, reflected by the localization of ADF, profilin and villin, are fundamental for gravity-sensing and polar growth in characean rhizoids. Planta **219:** 379–388

Buchwald G, Friebel A, Galan JE, Hardt WD, Wittinghofer A, Scheffzek K (2002) Structural basis for the reversible activation of a Rho protein by the bacterial toxin SopE. EMBO J **21:** 3286–3295

Büschges R, Hollricher K, Panstruga R, Simons G, Wolter M, Frijters A, van Daelen R, van der Lee T, Diergaarde P, Groenendijk J, et al (1997) The barley *Mlo* gene: a novel control element of plant pathogen resistance. Cell **88:** 695–705

Burridge K, Wennerberg K (2004) Rho and Rac take center stage. Cell **116:** 167–179

Carlier MF, Laurent V, Santolini J, Melki R, Didry D, Xia GX, Hong Y, Chua NH, Pantaloni D (1997) Actin depolymerizing factor (ADF/cofilin) enhances the rate of filament turnover: implication in actin-based motility. J Cell Biol **136:** 1307–1322

Chen CY, Wong EI, Vidali L, Estavillo A, Hepler PK, Wu HM, Cheung AY (2002) The regulation of actin organization by actin-depolymerizing factor in elongating pollen tubes. Plant Cell **14:** 2175–2190

Chen CYH, Cheung AY, Wu HM (2003) Actin-depolymerizing factor mediates Rac/Rop GTPase-regulated pollen tube growth. Plant Cell **15:** 237–249

Collins NC, Thordal-Christensen H, Lipka V, Bau S, Kombrink E, Qiu JL, Hückelhoven R, Stein M, Freialdenhoven A, Somerville SC, et al (2003) SNARE-protein-mediated disease resistance at the plant cell wall. Nature **425:** 973–977

Consonni C, Humphry ME, Hartmann HA, Livaja M, Durner J, Westphal L, Vogel J, Lipka V, Kemmerling B, Schulze-Lefert P, et al (2006) Conserved requirement for a plant host cell protein in powdery mildew pathogenesis. Nat Genet **38:** 716–720

Cooper JA (1987) Effects of cytochalasin and phalloidin on actin. J Cell Biol **105:** 1473–1478

DePina AS, Langford GM (1999) Vesicle transport: the role of actin filaments and myosin motors. Microsc Res Tech **47:** 93–106

Devoto A, Hartmann HA, Piffanelli P, Elliott C, Simmons C, Taramino G, Goh CS, Cohen FE, Emerson BC, Schulze-Lefert P, et al (2003) Molecular phylogeny and evolution of the plant-specific seven-transmembrane MLO family. J Mol Evol **56:** 77–88

Devoto A, Piffanelli P, Nilsson I, Wallin E, Panstruga R, von Heijne G, Schulze-Lefert P (1999) Topology, subcellular localization, and sequence diversity of the *Mlo* family in plants. J Biol Chem **274:** 34993–35004

Dong CH, Xia GX, Hong Y, Ramachandran S, Kost B, Chua NH (2001) ADF proteins are involved in the control of flowering and regulate F-actin organization, cell expansion, and organ growth in Arabidopsis. Plant Cell **13:** 1333–1346

Dos Remedios CG, Chhabra D, Kekic M, Dedova IV, Tsubakihara M, Berry DA, Nosworthy NJ (2003) Actin binding proteins: regulation of cytoskeletal microfilaments. Physiol Rev **83:** 433–473

Eichmann R, Schultheiss H, Kogel KH, Hückelhoven R (2004) The barley apoptosis suppressor homologue bax inhibitor-1 compromises nonhost penetration resistance of barley to the inappropriate pathogen *Blumeria graminis* f. sp *tritici*. Mol Plant Microbe Interact **17:** 484–490

Elliott C, Müller J, Miklis M, Bhat RA, Schulze-Lefert P, Panstruga R (2005) Conserved extracellular cysteine residues and cytoplasmic loop-loop interplay are required for functionality of the heptahelical MLO protein. Biochem J **385:** 243–254

Falk A, Feys BJ, Frost LN, Jones JDG, Daniels MJ, Parker JE (1999) EDS1, an essential component of R gene-mediated disease resistance in Arabidopsis has homology to eukaryotic lipases. Proc Natl Acad Sci USA **96:** 3292–3297

Feng Y, Liu Q, Xue Q (2006) Comparative study of rice and Arabidopsis actin-depolymerizing factors gene families. J Plant Physiol **163:** 69–79

Feys BJ, Moisan LJ, Newman MA, Parker JE (2001) Direct interaction between the Arabidopsis disease resistance signaling proteins, EDS1 and PAD4. EMBO J **20:** 5400–5411

Freialdenhoven A, Orme J, Lahaye T, Schulze-Lefert P (2005) Barley *Rom1* reveals a potential link between race-specific and nonhost resistance responses to powdery mildew fungi. Mol Plant Microbe Interact **18:** 291–299

Freialdenhoven A, Peterhänsel C, Kurth J, Kreuzaler F, Schulze-Lefert P (1996) Identification of genes required for the function of non-race-specific *mlo* resistance to powdery mildew in barley. Plant Cell **8:** 5–14

Gus-Mayer S, Naton B, Hahlbrock K, Schmelzer E (1998) Local mechanical stimulation induces components of the pathogen defense response in parsley. Proc Natl Acad Sci USA **95:** 8398–8403

Halterman D, Zhou FS, Wei FS, Wise RP, Schulze-Lefert P (2001) The MLA6 coiled-coil, NBS-LRR protein confers *AvrMla6*-dependent resistance specificity to *Blumeria graminis* f. sp *hordei* in barley and wheat. Plant J **25:** 335–348

Hauck P, Thilmony R, He SY (2003) A *Pseudomonas syringae* type III effector suppresses cell wall-based extracellular defense in susceptible Arabidopsis plants. Proc Natl Acad Sci USA **100:** 8577–8582

Hein I, Barciszewska-Pacak M, Hrubikova K, Williamson S, Dinesen M, Soenderby IE, Sundar S, Jarmolowski A, Shirasu K, Lacomme C (2005) VIGS-based functional characterization of genes associated with powdery mildew resistance in barley. Plant Physiol **138:** 2155–2164

Hückelhoven R, Dechert C, Kogel KH (2003) Overexpression of barley BAX inhibitor 1 induces breakdown of *mlo*-mediated penetration resistance to *Blumeria graminis*. Proc Natl Acad Sci USA **100:** 5555–5560

Humphry M, Consonni C, Panstruga R (2006) *mlo*-based powdery mildew immunity: silver bullet or simply non-host resistance? Mol Plant Pathol **7:** 605–610

Iriarte M, Cornelis GR (1998) YopT, a new Yersinia Yop effector protein, affects the cytoskeleton of host cells. Mol Microbiol **29:** 915–929

Jacobs AK, Lipka V, Burton RA, Panstruga R, Strizhov N, Schulze-Lefert P, Fincher GB (2003) An Arabidopsis callose synthase, GSL5, is required for wound and papillary callose formation. Plant Cell **15:** 2503–2513

Jedd G, Chua NH (2002) Visualization of peroxisomes in living plant cells reveals acto-myosin-dependent cytoplasmic streaming and peroxisome budding. Plant Cell Physiol **43:** 384–392

Jørgensen JH (1994) Genetics of powdery mildew resistance in barley. CRC Crit Rev Plant Sci **13:** 97–119

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

Kim MC, Panstruga R, Elliott C, Müller J, Devoto A, Yoon HW, Park HC, Cho MJ, Schulze-Lefert P (2002) Calmodulin interacts with MLO protein to regulate defence against mildew in barley. Nature **416:** 447–450

Kobayashi I, Hakuno H (2003) Actin-related defense mechanism to reject penetration attempt by a non-pathogen is maintained in tobacco BY-2 cells. Planta **217:** 340–345

Kobayashi I, Kobayashi Y, Hardham AR (1994) Dynamic reorganization of microtubules and microfilaments in flax cells during the resistance response to flax rust infection. Planta **195:** 237–247

Kobayashi Y, Kobayashi I, Funaki Y, Fujimoto S, Takemoto T, Kunoh H (1997a) Dynamic reorganization of microfilaments and microtubules is necessary for the expression of non-host resistance in barley coleoptile cells. Plant J **11:** 525–537

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

Lipka V, Dittgen J, Bednarek P, Bhat R, Wiermer M, Stein M, Landtag J, Brandt W, Rosahl S, Scheel D, et al (2005) Pre- and postinvasion defenses both contribute to nonhost resistance in Arabidopsis. Science **310:** 1180–1183

Lipka V, Panstruga R (2005) Dynamic cellular responses in plant-microbe interactions. Curr Opin Plant Biol **8:** 625–631

Maciver SK, Hussey PJ (2002) The ADF/cofilin family: actin-remodeling proteins. Genome Biol **3:** 1–12

Mano S, Nakamori C, Hayashi M, Kato A, Kondo M, Nishimura M (2002) Distribution and characterization of peroxisomes in arabidopsis by visualization with GFP: dynamic morphology and actin-dependent movement. Plant Cell Physiol **43:** 331–341

Mathur J, Mathur N, Hülskamp M (2002) Simultaneous visualization of peroxisomes and cytoskeletal elements reveals actin and not microtubule-based peroxisome motility in plants. Plant Physiol **128:** 1031–1045

McGhie EJ, Hayward RD, Koronakis V (2004) Control of actin turnover by a Salmonella invasion protein. Mol Cell **13:** 497–510

Morejohn LC, Bureau TE, Molebajer J, Bajer AS, Fosket DE (1987) Oryzalin, a dinitroaniline herbicide, binds to plant tubulin and inhibits microtubule polymerization in vitro. Planta **172:** 252–264

Mori Y, Sato Y, Takamatsu S (2000) Molecular phylogeny and radiation time of Erysiphales inferred from the nuclear ribosomal DNA sequences. Mycoscience **41:** 437–447

Moriyama K, Iida K, Yahara I (1996) Phosphorylation of Ser-3 of cofilin regulates its essential function on actin. Genes Cells **1:** 73–86

Muskett PR, Kahn K, Austin MJ, Moisan LJ, Sadanandom A, Shirasu K, Jones JDG, Parker JE (2002) Arabidopsis *RAR1* exerts rate-limiting control of *R* gene-mediated defenses against multiple pathogens. Plant Cell **14:** 979–992

Nishimura MT, Stein M, Hou BH, Vogel JP, Edwards H, Somerville SC (2003) Loss of a callose synthase results in salicylic acid-dependent disease resistance. Science **301:** 969–972

Opalski KS, Schultheiss H, Kogel KH, Hückelhoven R (2005) The receptor-like MLO protein and the RAC/ROP family G-protein RACB modulate actin reorganization in barley attacked by the biotrophic powdery mildew fungus *Blumeria graminis* f. sp *hordei*. Plant J **41:** 291–303

Ouellet F, Carpentier E, Cope M, Monroy AF, Sarhan F (2001) Regulation of a wheat actin-depolymerizing factor during cold acclimation. Plant Physiol **125:** 360–368

Panstruga R (2003) Establishing compatibility between plants and obligate biotrophic pathogens. Curr Opin Plant Biol **6:** 320–326

Panstruga R (2005) Serpentine plant MLO proteins as entry portals for powdery mildew fungi. Biochem Soc Trans **33:** 389–392

Panstruga R, Schulze-Lefert P (2003) Corruption of host seven-transmembrane proteins by pathogenic microbes: a common theme in animals and plants? Microbes Infect **5:** 429–437

Peart JR, Lu R, Sadanandom A, Malcuit I, Moffett P, Brice DC, Schauser L, Jaggard DAW, Xiao SY, Coleman MJ, et al (2002) Ubiquitin ligase-associated protein SGT1 is required for host and nonhost disease resistance in plants. Proc Natl Acad Sci USA **99:** 10865–10869

Pederson KJ, Krall R, Riese MJ, Barbieri JT (2002) Intracellular localization modulates targeting of ExoS, a type III cytotoxin, to eukaryotic signalling proteins. Mol Microbiol **46:** 1381–1390

Peterhänsel C, Freialdenhoven A, Kurth J, Kolsch R, Schulze-Lefert P (1997) Interaction analyses of genes required for resistance responses to powdery mildew in barley reveal distinct pathways leading to leaf cell death. Plant Cell **9:** 1397–1409

Reumann S (2004) Specification of the peroxisome targeting signals type 1 and type 2 of plant peroxisomes by bioinformatics analyses. Plant Physiol **135:** 783–800

Russo VM, Bushnell WR (1989) Responses of barley cells to puncture by microneedles and to attempted penetration by *Erysiphe graminis* f. sp. *hordei*. Can J Bot **67:** 2912–2921

Salmeron JM, Staskawicz BJ (1993) Molecular characterization and Hrp dependence of the avirulence gene avrpro from *Pseudomonas syringae* pv tomato. Mol Gen Genet **239:** 6–16

Schmelzer E (2002) Cell polarization, a crucial process in fungal defence. Trends Plant Sci **7:** 411–415

Schultheiss H, Dechert C, Kogel KH, Hückelhoven R (2002) A small GTP-binding host protein is required for entry of powdery mildew fungus into epidermal cells of barley. Plant Physiol **128:** 1447–1454

Schultheiss H, Dechert C, Kogel KH, Hückelhoven R (2003) Functional analysis of barley RAC/ROP G-protein family members in susceptibility to the powdery mildew fungus. Plant J **36:** 589–601

Schulze-Lefert P (2004) Knocking on heaven's wall: pathogenesis of and resistance to biotrophic fungi at the cell wall. Curr Opin Plant Biol **7:** 377–383

Schweizer P, Christoffel A, Dudler R (1999) Transient expression of members of the *germin*-like gene family in epidermal cells of wheat confers disease resistance. Plant J **20:** 540–552

Shimada C, Lipka V, O'Connel R, Okuno T, Schulze-Lefert P, Takano Y (2006) Nonhost resistance in *Arabidopsis-Colletotrichum* interactions acts at the cell periphery and requires actin filament function. Mol Plant Microbe Interact **19:** 270–279

Škalamera D, Heath MC (1998) Changes in the cytoskeleton accompanying infection-induced nuclear movements and the hypersensitive response in plant cells invaded by rust fungi. Plant J **16:** 191–200

Smertenko AP, Jiang CJ, Simmons NJ, Weeds AG, Davies DR, Hussey PJ (1998) Ser6 in the maize actin-depolymerizing factor, ZmADF3, is phosphorylated by a calcium-stimulated protein kinase and is essential for the control of functional activity. Plant J **14:** 187–193

Takahashi A, Casais C, Ichimura K, Shirasu K (2003) HSP90 interacts with RAR1 and SGT1 and is essential for RPS2-mediated disease resistance in *Arabidopsis*. Proc Natl Acad Sci USA **100:** 11777–11782

Takemoto D, Hardham AR (2004) The cytoskeleton as a regulator and target of biotic interactions in plants. Plant Physiol **136:** 3864–3876

Takemoto D, Jones DA, Hardham AR (2003) GFP-tagging of cell components reveals the dynamics of subcellular re-organization in response to infection of Arabidopsis by oomycete pathogens. Plant J **33:** 775–792

Theriot JA (1997) Accelerating on a treadmill: ADF/cofilin promotes rapid actin filament turnover in the dynamic cytoskeleton. J Cell Biol **136:** 1165–1168

Timonen S, Peterson RL (2002) Cytoskeleton in mycorrhizal symbiosis. Plant Soil **244:** 199–210

Trujillo M, Troeger M, Niks RE, Kogel KH, Hückelhoven R (2004) Mechanistic and genetic overlap of barley host and non-host resistance to *Blumeria graminis*. Mol Plant Pathol **5:** 389–396

van der Biezen EA, Jones JDG (1998) Plant disease-resistance proteins and the gene-for-gene concept. Trends Biochem Sci **23:** 454–456

Wolter M, Hollricher K, Salamini F, Schulze-Lefert P (1993) The *mlo* resistance alleles to powdery mildew infection in barley trigger a developmentally controlled defense mimic phenotype. Mol Gen Genet **239:** 122–128

Yun BW, Atkinson HA, Gaborit C, Greenland A, Read ND, Pallas JA, Loake GJ (2003) Loss of actin cytoskeletal function and EDS1 activity, in combination, severely compromises non-host resistance in Arabidopsis against wheat powdery mildew. Plant J **34:** 768–777

Zhou FS, Kurth JC, Wei FS, Elliott C, Vale G, Yahiaoui N, Keller B, Somerville S, Wise R, Schulze-Lefert P (2001) Cell-autonomous expression of barley *Mla1* confers race-specific resistance to the powdery mildew fungus via a *Rar1*-independent signaling pathway. Plant Cell **13:** 337–350

Zierold U, Scholz U, Schweizer P (2005) Transcriptome analysis of *mlo*-mediated resistance in the epidermis of barley. Mol Plant Pathol **6:** 139–151