Supporting Information

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SI Materials and Methods

***Arabidopsis* Growth Conditions and Transformation.** *Arabidopsis* seeds were sown on soil (Pro-Mix HP; Premier Horticulture) and stratified for 3 d at 4 °C. Plants were grown in growth chambers (Conviron) with a 12-h photoperiod at light intensities of 120 μM m^{-2} s^{-1} in the 400- to 700-nm range, 70% relative humidity, and temperatures of 22 °C during the light period and 21 °C during the dark period. The PEN3 promotor-PEN3-GFP transgenic line and *pen3-3* (SALK_110926) (1) mutant line used in this study are described in ref. 2. Transformation of *Arabidopsis* was carried out by the floral dip method (3).

Powdery Mildew and Bacterial Inoculations. *Blumeria graminis* f. sp. *hordei* (*Bgh*) race CR3 was maintained on barley variety CI-16138 (AlgerianS). Inoculations were carried out as previously described (4). *Escherichia coli* carrying *Discosoma* sp. red fluorescent protein (dsRED) in the plasmid pGEM-T easy were grown overnight in LB medium, pelleted by centrifugation for 2 min at 3,000 × *g*, and resuspended in H_2O at 10^8 cfu/mL. The bacterial suspension was infiltrated into leaves using a 1-mL needleless syringe, and inoculated leaves were imaged at 24 h postinoculation (hpi) by confocal microscopy as described below.

Pharmacological Inhibitor and TAMRA-flg22 Stock Solutions and Handling. Pharmacological inhibitors were prepared as stock solutions in DMSO at the following concentrations: cytochalasin E (AG Scientific), 2 mg/mL; latrunculin B (Sigma), 10 mM; and oryzalin (Sigma), 100 mM. All DMSO stocks were stored at −20 °C. Brefeldin A (Sigma) was prepared as a stock solution at 10 mg/mL in 100% EtOH and stored at 4 °C. Monensin (Sigma) was prepared as a stock solution at 10 mM in 100% EtOH and stored at −20 °C. Colchicine was prepared as a stock solution at 40 mg/mL in H_2O and stored at −20 °C. Cycloheximide (CHX) was prepared as a stock solution at 5 mg/mL in H_2O and stored at 4 °C; 5-TAMRA-flg22 peptide was synthesized by Elim Biopharmaceuticals at a purity level of ≥70%. Stocks were prepared by dissolving the peptide in H_2O at a concentration of 10 mM, and they were stored at −20 °C and subsequently diluted in H_2O to the specified concentration for experiments.

CHX Validation. For CHX validation, leaves from 3-wk-old *Arabidopsis* Col-0 plants were syringe-infiltrated with H_2O or 5 μg/mL CHX. After 1 h, treated leaves were syringe-infiltrated with 100 μCi/mL TRAN35S metabolic labeling reagent (MP Biomedicals). Treated leaves were collected at 4, 12, and 24 h after CHX/H_2O treatment, and proteins were extracted by freezing leaf tissue (one leaf per sample) in liquid N_2, grinding to powder, and homogenizing in 100 μL 2× SDS/PAGE sample buffer. Samples were centrifuged for 10 min at 15,000 × *g* at 4 °C to pellet cell debris, and supernatants were boiled for 10 min; 25 μL each protein sample were separated on a 4–15% gradient SDS gel (Bio-Rad). The gel was stained with Gelcode Blue Reagent (Thermo Fisher), photographed, and exposed to autoradiography film (Thermo Fisher) for 48 h at −80 °C. The film was developed using a Kodak X-omat 2000 Developer.

Bacterial Multiplication Assays. Bacteria for plant inoculations were grown to midlog phase in 10-mL liquid cultures. *E. coli* DH5α was grown in LB medium (5), and *Pseudomonas syringae* pv. *tomato* DC3000 *hrcC* was grown in low-salt (5 g/L) LB amended with 60 mg/L rifampicin. Bacterial cultures were centrifuged at 3,000 × *g* and resuspended in sterile water to $OD_{600} = 0.002$ (10^6 cfu/mL). Bacterial suspensions were infiltrated into leaves of 3- to 4-wk-old plants using a needleless 1-mL syringe against the abaxial leaf surface. After inoculation, plants were left uncovered until leaves were no longer water-soaked and then covered with a humidity dome for the remainder of the experiment. Bacterial populations in the apoplast were determined at days 0, 3, and 5 using a previously described method (6). A correction factor was calculated to equalize day 0 values, and this correction factor was used to normalize days 3 and 5 values for comparison. All bacterial multiplication assays were repeated three times with similar results.

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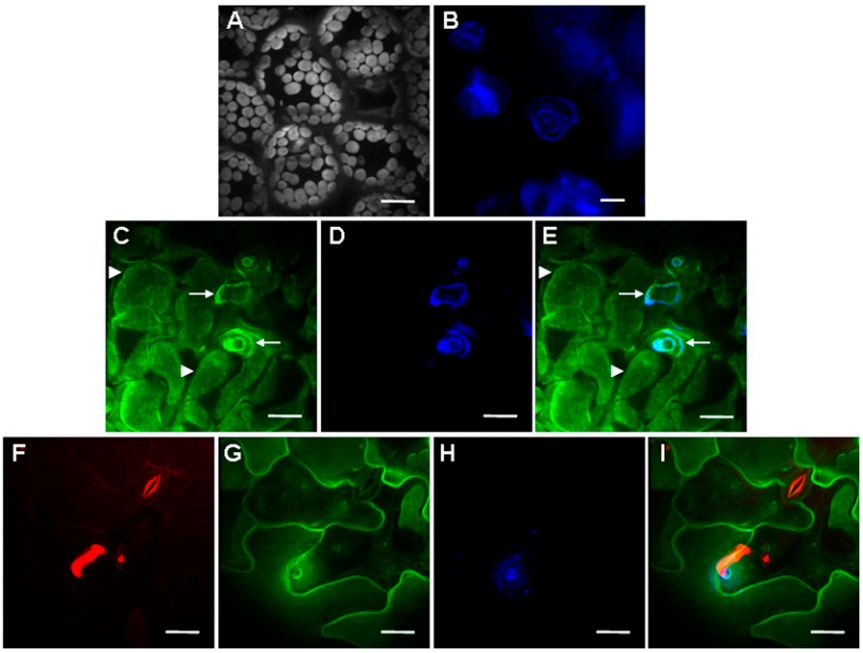

Fig. S1. PEN3-GFP and callose colocalize at focal accumulations (FAs) in flg22- and *Bgh*-treated leaves. (*A*) Confocal image of the mesophyll cell layer of a Col WT leaf 24 h after treatment with 5 μM flg22. No autofluorescent rings or bull's eye structures were observed. (*B*) Aniline blue-stained callose deposited 24 h after syringe infiltration of WT leaves with 5 μM flg22. (*C*) Localization of PEN3-GFP in mesophyll cells 24 h after syringe infiltration of 5 μM flg22. (*D*) Localization of sirofluor-stained callose in mesophyll cells 24 h after syringe infiltration of 5 μM flg22. (*E*) Overlay of *C* and *D* showing colocalization of PEN3-GFP and callose at flg22-induced FAs. (*F*) Propidium iodide-stained *Bgh* appressorium. Autofluorescence of stomatal guard cells is also visible in this channel. (*G*) Localization of PEN3-GFP in epidermal cells 24 h after inoculation with *Bgh*. (*H*) Localization of sirofluor-stained callose in epidermal cells 24 h after inoculation with *Bgh*. (*I*) Overlay of *F*–*I* showing colocalization of PEN3-GFP and callose at the site of attempted penetration by *Bgh*. Arrowheads in *C* and *E* indicate PEN3-GFP–labeled plasma membrane (PM) outlines of leaf mesophyll cells. Arrows in *C* and *E* indicate rings of PEN3-GFP FA. *A* and *C*–*I* are z-projected confocal images. (Scale bars: 20 μm.)

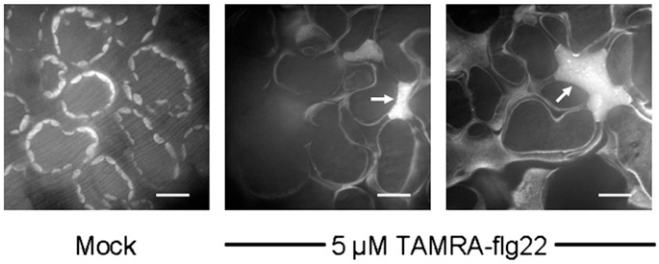

Fig. S2. Localization of TAMRA-flg22 fluorescent-labeled peptide after syringe infiltration. Z series was collected at 3 h after syringe infiltration of WT Col-0 leaves with either H_2O (mock) or 5 μM TAMRA-flg22 fluorescent peptide. Only autofluorescence from chloroplasts was observed in mock-treated samples. TAMRA-flg22 showed uneven accumulation and concentration in pools indicated by arrows. Note that chloroplast autofluorescence is relatively weak and not observed during visualization of fluorophores with strong fluorescent signals. All panels are z-projected confocal images. (Scale bars: 20 μm.)

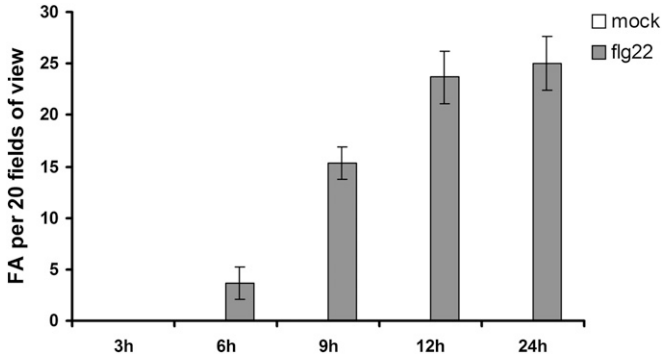

Fig. S3. Time course of PEN3-GFP FA after flg22 treatment. Ring-type FAs of PEN3-GFP were enumerated at 3, 6, 9, 12, and 24 h after syringe infiltration of 5 μM flg22. FAs were counted for 20 random microscope fields of view per leaf for three leaves at each time point. Error bars represent SD ($n = 3$).

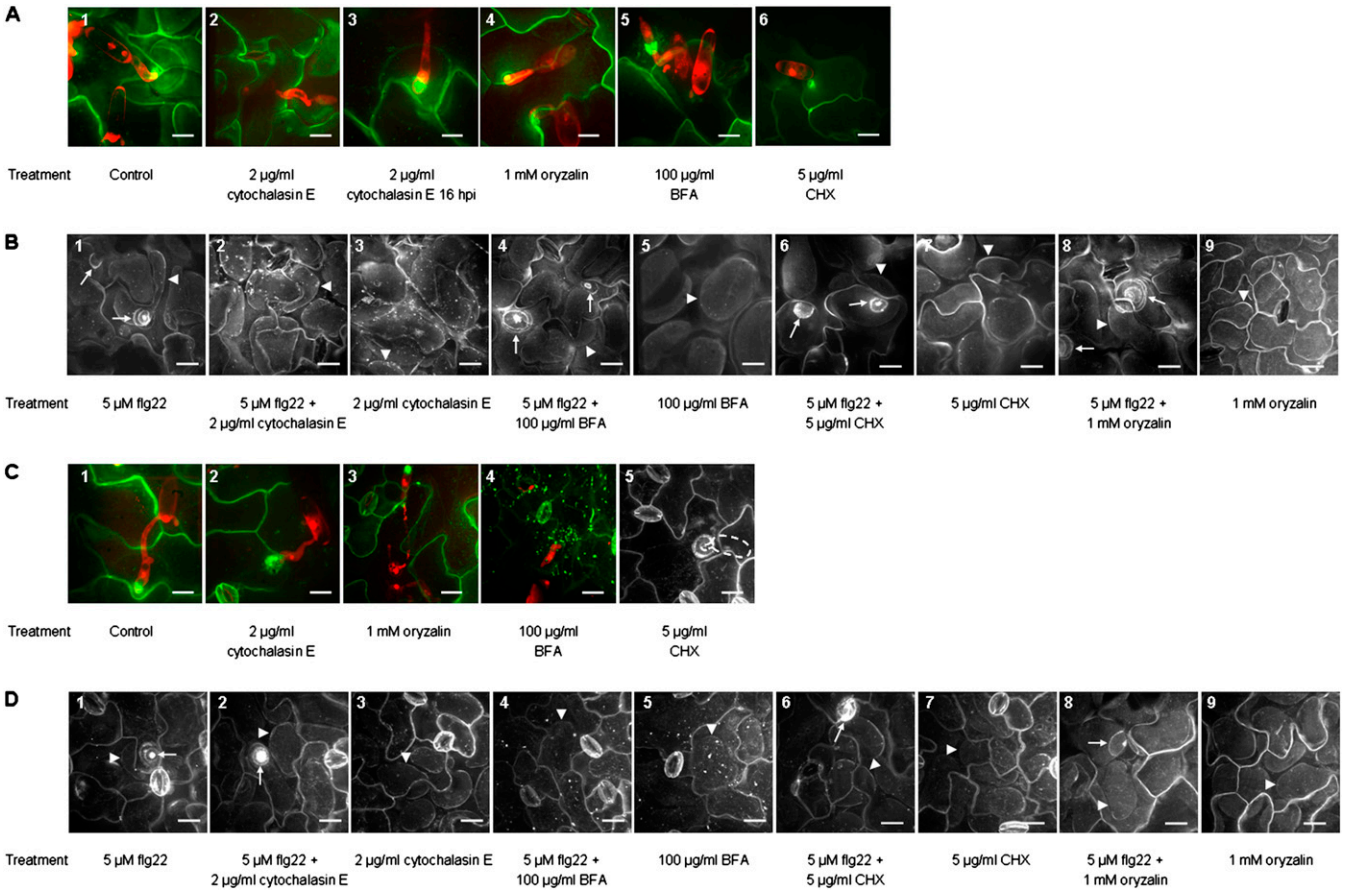

Fig. S4. Effects of pharmacological inhibitors on *Bgh*- and pathogen-associated molecular patterns-induced localization of PEN3 and PEN1. (*A*) Localization of PEN3-GFP in leaves syringe-infiltrated with 2 μg/mL cytochalasin E, 1 mM oryzalin, 100 μg/mL brefeldin A (BFA), 5 μg/mL CHX, or control treatments at 1 h before inoculation with *Bgh* (1, 2, and 4–6) or 16 hpi (3). Images were collected at 24 hpi (1, 2, and 4–6) or 40 hpi (3). Control treatments (H_2O + solvent) were performed for each inhibitor, and representative controls are shown. GFP fusions appear green. Propidium iodide-stained fungal structures appear red. (*B*) Localization of PEN3-GFP 24 h after syringe infiltration with 5 μM flg22 (1), 2 μg/mL cytochalasin E (3), 100 μg/mL BFA (5), 5 μg/mL CHX (7), or 1 mM oryzalin (9) alone or after coinfiltration with flg22 + cytochalasin E (2), flg22 + BFA (4), flg22 + CHX (6), or flg22 + oryzalin (8). Arrowheads indicate PEN3-GFP–labeled PM outlines of leaf mesophyll cells. Arrows indicate rings of PEN3-GFP FA. (*C*) Localization of GFP-PEN1 in leaves syringe-infiltrated with 2 μg/mL cytochalasin E, 1 mM oryzalin, 100 μg/mL BFA, 5 μg/mL CHX, or control treatments at 1 h before inoculation with *Bgh*. Images were collected at 24 hpi. Control treatments (H_2O + solvent) were performed for each inhibitor, and representative controls are shown. In 1–4, GFP fusions appear green, and propidium iodide-stained fungal structures appear red. In 5, the dashed white outline indicates the position of the *Bgh* conidiospore. Note that, in 4, no FA of GFP-PEN1 is observed at the tip of the appressorium, and in 5, GFP-PEN1 FA appears near the tip of the primary germ tube. (*D*) Localization of GFP-PEN1 24 h after syringe infiltration with 5 μM flg22 (1), 2 μg/mL cytochalasin E (3), 100 μg/mL BFA (5), 5 μg/mL CHX (7), or 1 mM oryzalin (9) alone or after coinfiltration with flg22 + cytochalasin E (2), flg22 + BFA (4), flg22 + CHX (6), or flg22 + oryzalin (8). Arrowheads indicate GFP-PEN1–labeled PM outlines of leaf mesophyll cells. Arrows indicate rings of GFP-PEN1 FA. All image panels are z-projected confocal images. (Scale bars: 20 μm.)

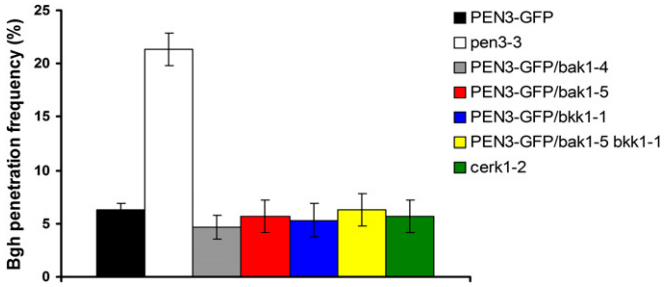

Fig. S5. *Bgh* penetration frequencies on *bak1* and *bkk1* mutants. Penetration frequencies on PEN3-GFP (black), *pen3-3* (white), PEN3-GFP/*bak1-4* (gray), PEN3-GFP/*bak1-5* (red), PEN3-GFP/*bkk1-1* (blue), PEN3-GFP/*bak1-5 bkk1-1* (yellow), and *cerk1-2* (green) were determined by scoring for presence or absence of haustoria at 100 infection sites per leaf for three leaves per plant line 48 hpi. Error bars represent SD ($n = 3$).

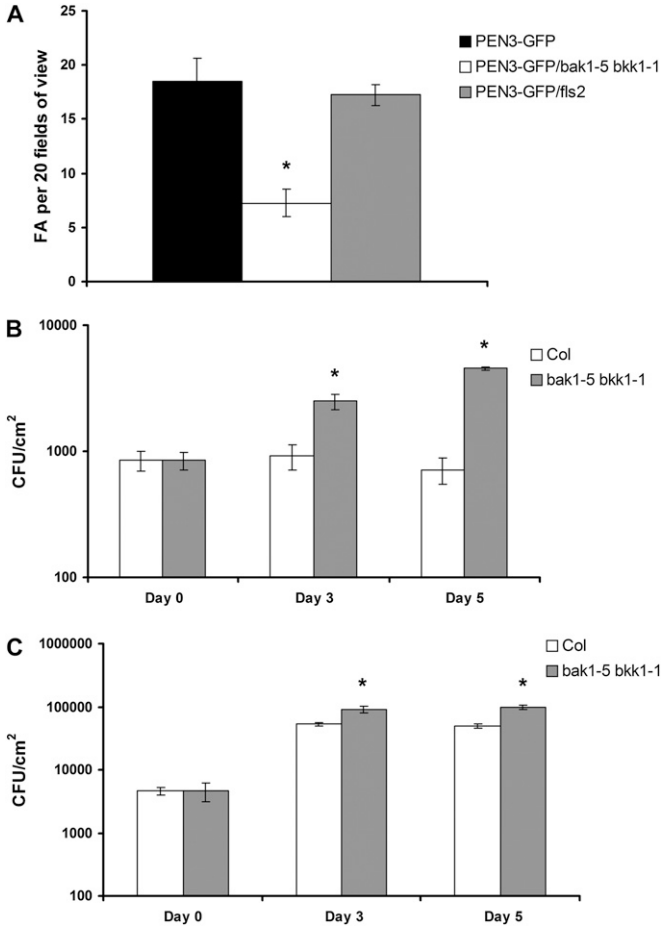

Fig. S6. Bacteria-induced PEN3-GFP FA in *bak1-5 bkk1-1* and *fls2* mutants and bacterial multiplication in *bak1-5 bkk1-1*. (*A*) Quantitative display of the frequency of PEN3-GFP FAs in WT background (PEN3-GFP; black), *bak1-5 bkk1-1* double mutant (white), or *fls2* mutant (gray) leaves at 24 h after inoculation with 10^8 cfu/mL *E. coli* DH5α bacteria. FAs were enumerated for 20 random microscope fields of view per leaf for three leaves per line. Error bars represent SD. Asterisk indicates significant difference ($P < 0.05$; Tukey honestly significant difference test). (*B*) Multiplication of *E. coli* DH5α in WT Col-0 and *bak1-5 bkk1-1* mutant leaves. Bacterial populations were extracted at 0, 3, and 5 d after syringe infiltration with 10^6 cfu/mL bacterial suspension in H_2O. Population numbers were normalized to equalize day 0 values for comparison. Error bars represent SEM. Asterisks indicate significant differences ($P < 0.05$; Student *t* test). (*C*) Multiplication of *P. syringae* pv. *tomato* DC3000 *hrcC* in WT Col-0 and *bak1-5 bkk1-1* mutant leaves. Bacterial populations were extracted at 0, 3, and 5 d after syringe infiltration with 10^6 cfu/mL bacterial suspension in H_2O. Population numbers were normalized to equalize day 0 values for comparison. Error bars represent SEM. Asterisks indicate significant differences ($P < 0.05$; Student *t* test).

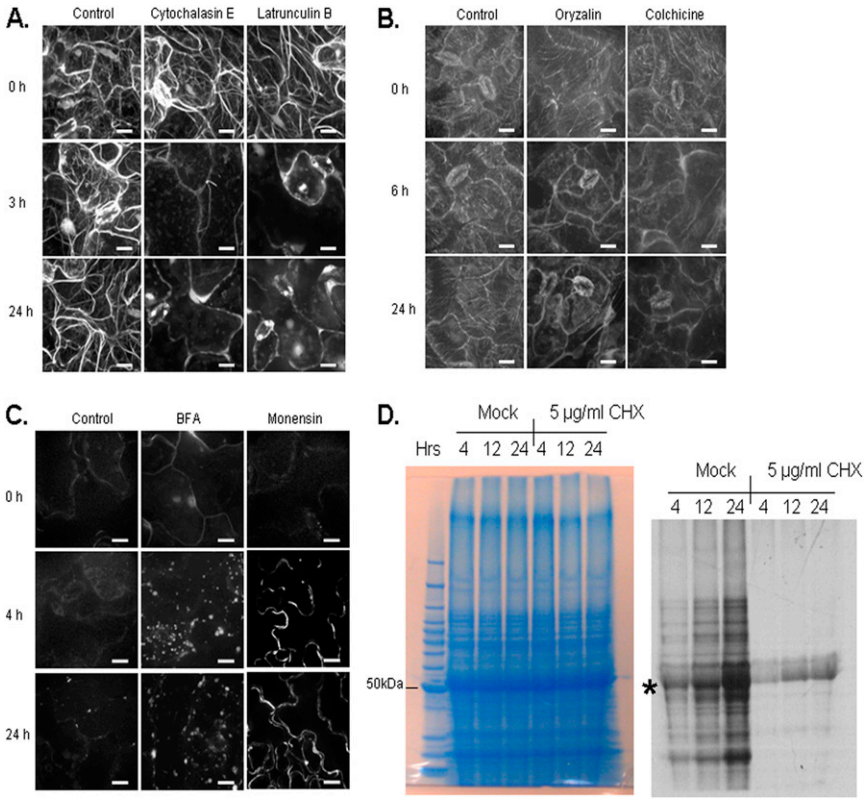

Fig. S7. Efficacy of pharmacological inhibitors. (*A*) Actin filaments visualized in plants expressing GFP-talin after syringe infiltration with (*Center*) 2 μg/mL cytochalasin E, (*Right*) 20 μM latrunculin B, or (*Left*) a control (H_2O + solvent) treatment. Images were collected at (*Top*) 0, (*Middle*) 3, and (*Bottom*) 24 h after inhibitor treatment. (*B*) Microtubules visualized in plants expressing GFP–β-tubulin fusion protein after syringe infiltration with (*Center*) 1 mM oryzalin, (*Right*) 40 μg/mL colchicine, or (*Left*) a control (H_2O + solvent) treatment. Images were collected at (*Top*) 0, (*Middle*) 6, and (*Bottom*) 24 h after inhibitor treatment. (*C*) Localization of secretion-targeted GFP variant in leaves syringe-infiltrated with (*Center*)100 μg/mL BFA, (*Right*) 1 μM monensin, or (*Left*) a control (H_2O + solvent) treatment. Images were collected (*Top*) 0, (*Middle*) 4, and (*Bottom*) 24 h after inhibitor treatment. Note that secretion-targeted GFP is poorly visualized in control-treated samples because of low apoplastic pH, but it accumulates intracellularly in the presence of BFA and monensin. All panels are z-projected confocal images. (Scale bars: 20 μm.) (*D*) Effect of 5 μg/mL CHX on incorporation of ^{35}S-labeled methionine and cysteine into newly synthesized proteins. (*Left*) Gelcode Blue-stained gel image and (*Right*) autoradiography are shown. Leaves infiltrated with 5 μg/mL CHX or H_2O were collected at the times indicated for protein extraction. Asterisk indicates the position of the large subunit of ribulose bisphosphate carboxylase, an abundant leaf protein synthesized in chloroplasts. Synthesis of proteins by chloroplast and mitochondrial ribosomes has previously been shown to be insensitive to CHX (1, 2). Note that a minimum of 2 h passes before conidia start to germinate, producing primary germ tubes that are incapable of forming haustoria. By about 6 h after inoculation, appressorial germ tubes have formed, and by 14 hpi, immature haustoria can be observed, suggesting that the plant cuticle and cell wall are breached by *Bgh* in this time frame (3). Thus, by the time that *Bgh* initiates an attack on the plant, the inhibitors used in this study will be fully effective.

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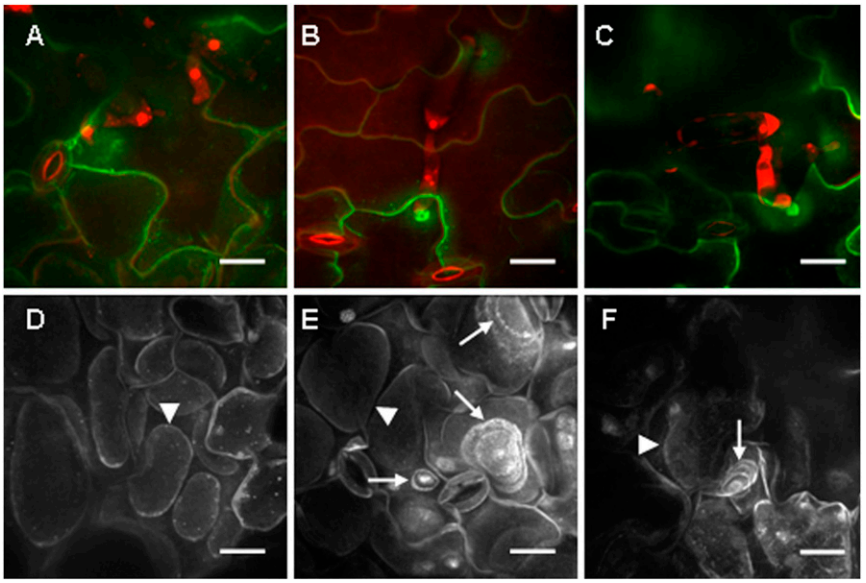

Fig. S8. Effects of latrunculin B, colchicine, and monensin on PEN3 localization. (*A*) Localization of PEN3-GFP in leaves syringe-infiltrated with 20 μM latrunculin B 1 h before inoculation with *Bgh*. (*B*) Localization of PEN3-GFP in leaves syringe-infiltrated with 40 μg/mL colchicine 1 h before inoculation with *Bgh*. (*C*) Localization of PEN3-GFP in leaves syringe-infiltrated with 1 μM monensin 1 h before inoculation with *Bgh*. (*D*) Localization of PEN3-GFP in leaves coinfiltrated with 20 μM latrunculin B + 5 μM flg22. (*E*) Localization of PEN3-GFP in leaves coinfiltrated with 40 μg/mL colchicine + 5 μM flg22. (*F*) Localization of PEN3-GFP in leaves coinfiltrated with 1 μM monensin + 5 μM flg22. Images were collected at 24 h after *Bgh* inoculation or flg22 infiltration. In *A*–*C*, PEN3-GFP appears green, and propidium iodide-stained fungal structures appear red. Note that accumulation of PEN3-GFP at the tip of the fungal appressorium is not observed in *A*. In *D*–*F*, arrowheads indicate PEN3-GFP–labeled PM outlines of leaf mesophyll cells, and in *E* and *F*, arrows indicate rings of PEN3-GFP FA. All panels are z-projected confocal images. (Scale bars: 20 μm.)

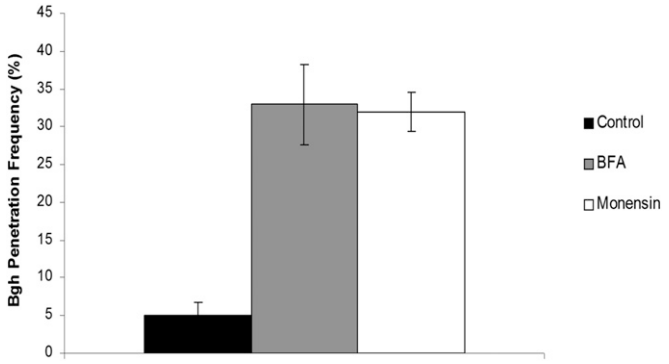

Fig. S9. Vesicle trafficking inhibitors impair penetration defense. Penetration frequency of *Bgh* on leaves syringe-infiltrated with 100 μg/mL BFA, 1 μM monensin, or a control (H_2O + solvent) treatment 1 h before inoculation with the fungus. Penetration frequency was determined by scoring for presence or absence of haustoria at 100 infection sites per leaf for three leaves per plant line 48 hpi. Error bars represent SD (n = 3). The experiment was repeated three times with similar results.