HopW1 from *Pseudomonas syringae* Disrupts the Actin Cytoskeleton to Promote Virulence in Arabidopsis

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**Abstract**

A central mechanism of virulence of extracellular bacterial pathogens is the injection into host cells of effector proteins that modify host cellular functions. HopW1 is an effector injected by the type III secretion system that increases the growth of the plant pathogen *Pseudomonas syringae* on the Columbia accession of Arabidopsis. When delivered by *P. syringae* into plant cells, HopW1 causes a reduction in the filamentous actin (F-actin) network and the inhibition of endocytosis, a known actin-dependent process. When directly produced in plants, HopW1 forms complexes with actin, disrupts the actin cytoskeleton and inhibits endocytosis as well as the trafficking of certain proteins to vacuoles. The C-terminal region of HopW1 can reduce the length of actin filaments and therefore solubilize F-actin *in vitro*. Thus, HopW1 acts by disrupting the actin cytoskeleton and the cell biological processes that depend on actin, which in turn are needed for restricting *P. syringae* growth in Arabidopsis.

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**Introduction**

Plants that are infected with foliar bacterial pathogens can mount a multilayered response, the success of which is shaped by the perception of pathogen-derived molecules and the ability of the pathogen to disrupt host responses. Essential for understanding dynamic host-pathogen interactions is the identification of critical components of the host defense machinery and the biochemical mechanism by which bacterial factors interfere with host functions. At least two types of molecules from plant pathogenic bacteria can trigger defenses: conserved patterns (pathogen-associated molecular patterns, PAMPs) that bind to cell surface pattern receptors and more variable effectors that are injected by bacteria into the plants [1]. The perception by plants of some bacterial effectors occurs through the deployment of intracellular immune complexes [1]. A major consequence of bacterial effector activities is to promote virulence, which can occur when plants lack immune receptors for particular effectors. Some of the best-studied effectors are those that form the set of proteins introduced into plants through a type three secretion system (TTSS) [2].

*Pseudomonas syringae* is an extracellular pathogen that causes several types of foliar disease in agriculturally important plant species [3]. In the model plants *Arabidopsis thaliana* and *Nicotiana benthamiana*, pattern receptors and hormonal signals contribute quantitatively to defense against virulent *P. syringae* [1,4,5]. However, some *P. syringae* effectors can inhibit the action of receptors, accumulation and/or action of hormone/defense signals and other processes important for quantitative defense activation [6,7,8].

An emerging area of research has focused on cytoskeleton components as specific virulence targets of *P. syringae*. At least one effector, HopZ1a, acetylates tubulin *in vitro* and causes microtubule disruption *in planta* [9]. Treatment of plants with cytochalasins, compounds that prevent actin polymerization, increases the ability of several fungal pathogens to penetrate plant tissue [10,11]. Infection of Arabidopsis with *P. syringae* or treatment with PAMPs induces dynamic changes in actin filament density and bundling [12,13]. Application of a drug that depolymerizes filamentous actin (F-actin) causes increased growth of *P. syringae* in *planta* [12]. These observations raise the possibility that specific effectors target the actin cytoskeleton to disrupt actin-dependent immune responses. In plants, the actin cytoskeleton is important for various cell biological processes [14] that may be important for immune signaling, including endocytosis and the trafficking of some vacuolar proteins [15]. While specific *P. syringae* effectors that target actin have not yet been reported, injected virulence factors from several mammalian pathogens have been shown to directly interact with actin or modify cellular components that regulate actin [16,17,18].

Nearly a decade of research on effectors has uncovered several examples of *P. syringae* TTSS effectors that can interact with multiple host proteins. Whereas some interactions trigger immunity, others promote virulence [8,19]. The HopW1 gene, which resides on a multicopy plasmid in *P. syringae* pv. *maculicola* strain...
Actin cytoskeleton. The effects of HopW1 can be mimicked using a drug that inhibits actin polymerization. Thus, this work establishes a direct mechanism for pathogen disruption of the actin cytoskeleton and implicates actin-dependent events as important for controlling pathogen growth during infection.

Results

Actin Co-purifies with HopW1

To find components of HopW1-containing complexes, we used LC-MS/MS to identify proteins that co-purified with HopW1-HA that was transiently expressed in N. benthamiana. Thirteen unique peptides derived from actin (covering 47% of protein) were identified from a co-precipitating band of 43 kDa that was absent in the control immunoprecipitation (IP). We confirmed that HopW1 and actin formed a complex using IP and immunoblotting of extracts from N. benthamiana and Arabidopsis that transiently expressed HopW1-HA (Figure 1). HopW1 does not have any known binding motifs or sequences that could help predict its activity. However, the high amount of actin in HopW1 could be a major target of HopW1 that disrupts actin filaments in planta.

HopW1 Disrupts Actin Filaments during Infection

In eukaryotic cells, actin exists as both dynamic filaments (F-actin) and as a large pool of unpolymerized actin [23]. We used Arabidopsis Col expressing Lifeact-GFP (green fluorescent protein) that binds F-actin [24,25,26] to visualize the actin cytoskeleton (Figure 2A). We imaged F-actin by confocal microscopy in Col/Lifeact-GFP seedlings infected with PDC3000 containing HopW1 (PDC3000/HopW1) or a vector control (pME6012). We used ectopic expression in a heterologous P. syringae DC3000 containing HopW1/HopW1-HA (Figure 2A). We imaged F-actin by confocal microscopy in Col/Lifeact-GFP seedlings infected with PDC3000 containing HopW1 (PDC3000/HopW1) or a vector control (pME6012). We used ectopic expression in a heterologous P. syringae DC3000 containing HopW1/HopW1-HA (Figure 2A). We imaged F-actin by confocal microscopy in Col/Lifeact-GFP seedlings infected with PDC3000 containing HopW1 (PDC3000/HopW1) or a vector control (pME6012). We used ectopic expression in a heterologous P. syringae DC3000 containing HopW1/HopW1-HA (Figure 2A).

The Virulence Effect of HopW1 Is Phenocopied by Latrunculin B

If the actin cytoskeleton is a major target of HopW1, a prediction is that pharmacological disruption of the actin cytoskeleton would phenocopy the virulence effect of HopW1. Indeed, LatB treatment increased the growth of PDC3000 on Arabidopsis [12], an effect we also observed (Figure 2C). LatB had the same magnitude of effect as HopW1 to increase PDC3000 growth on Col Arabidopsis (Figure 2C). PDC3000/HopW1 grew slightly more than PDC3000/vector in LatB-treated plants. However, the additive effect was very small and might not be biologically meaningful. The results show that the net effects of HopW1 and LatB are similar, consistent with disruption of actin being responsible for increased pathogen growth.

HopW1 Alone Disrupts the Actin Cytoskeleton in Plant Cells

We directly expressed HopW1 in plant cells to see if it is sufficient to affect the actin cytoskeleton. When transiently co-expressed in N. benthamiana with Lifeact-GFP, HopW1-RFP (red fluorescent protein) disrupted F-actin to such a degree that HopW1 was only detected in cells with very little Lifeact-GFP (Figure 3A). This pattern was seen in all cells with detectable HopW1. At earlier times (16–24 h after Agroinfiltration), no fluorescent protein signals were detected. Plant cells in which the actin cytoskeleton was labeled with Lifeact-GFP lacked detectable HopW1-RFP signals. In contrast, dense F-actin was present in cells with control mCherry (Figure 3A). When
detectable, HopW1-RFP accumulated in patches at the periphery of cells (Figure 3A). Similar patterns were observed when HopW1-RFP was transformed alone into N. benthamiana leaves without Lifeact-GFP (not shown). Localization of HopW1 in patches may result from disorganization of the actin cytoskeleton at the time we can detect HopW1. During the infection of Arabidopsis with PtoDC3000/HopW1 or LatB treatment, most Lifeact-GFP marked filaments also disappeared from the cell interiors and the signal remained at the periphery (Figure 3A). Similarly, in protoplasts from Arabidopsis Lifeact-GFP plants transiently expressing HopW1-CFP (cyan fluorescent protein), the F-actin cytoskeleton was absent 15 h after transformation and HopW1-CFP was found in patches mostly along cell border (Figure 3B). Only protoplasts with undetectable HopW1-CFP signal had an intact actin cytoskeleton (not shown). These data show that HopW1 is sufficient to disrupt actin filaments in plant cells.

HopW1-C Disrupts F-actin In Vitro

To test whether the effect of HopW1 on the actin cytoskeleton observed in planta is direct, we assayed the activity of recombinant HopW1 on actin filaments in vitro. To evaluate the ratio of soluble to F-actin, we performed sedimentation assays after 30 min. incubation of pre-assembled F-actin with recombinant His-HopW1-C (HopW1107-774) [27]. We used a truncated version of HopW1 because full-length protein was insoluble. We validated that actin co-purified from plants with full length and the C-terminal domain, but not with N-terminal domain, although HopW1-C accumulated in planta at much lower level than other variants (Figure S1). HopW1-C, but not E. coli extract or BSA, increased the amount of actin in the supernatant and simultaneously decreased actin in the pellet (Figure 4A), indicating a dose-dependent ability of HopW1-C to solubilize F-actin (Figure 4B).

Sedimentation assays were corroborated by visualizing changes in the distribution of actin filament lengths after 1 h incubation with a range of HopW1-C concentrations. There was a clear and statistically significant shift to smaller filament lengths as a function of the amount of HopW1-C added to preassembled F-actin (Figure 4C and 4D). These assays employed non-muscle actin. We did not detect disruption of muscle F-actin by HopW1-C (Figure S2): Thus, HopW1-C can directly disrupt non-muscle F-actin in vitro.

HopW1 Disrupts Actin-Dependent Protein Targeting

Disruption of the actin cytoskeleton may lead to disruption of intracellular trafficking that is essential for plant immunity [13,28,29,30]. As a first test of this possibility, we assessed HopW1’s effect on the trafficking of marker proteins. A functional
The actin cytoskeleton is needed for the trafficking of sporamin-GFP (SPO-GFP) and Arabidopsis aleurin-like protein-GFP (AALP-GFP) to the endoplasmic reticulum (ER) and/or vacuole [14] (ER localization of SPO-GFP occurs prior to its transport to the vacuole [14]). In the absence of HopW1, both reporter proteins individually transfected to Col protoplasts showed patterns consistent with ER and/or vacuole localization (Figure 5A, upper panels), as previously shown [14]. In contrast, in transgenic Arabidopsis protoplasts from a transgenic line that expresses Lifeact-GFP [26] were transfected with HopW1-CFP (lower panel) or without DNA (control, upper panel). Micrographs show Lifeact-GFP and HopW1-CFP 15 h after transfection. GFP/CFP fluorescence is shown in green/magenta, respectively, and chloroplast (chl) autofluorescence in blue. These experiments were repeated twice with similar results.

Control treatment with LatB to disrupt the actin cytoskeleton also caused similar punctate GFP patterns (Figure S3A), as previously reported [14]. Quantitation of the GFP patterns showed that HopW1 (and LatB) significantly disrupted the normal localization patterns of AALP-GFP and SPO-GFP, respectively (Figure 5B and S3B). Therefore, HopW1 prevents normal localization of proteins whose targeting depends on actin in a similar way as a drug that disrupts the actin cytoskeleton.

Figure 3. HopW1 disrupts the actin cytoskeleton when expressed in plant cells. Localization and effect of HopW1 on actin cytoskeleton was monitored in transiently transformed cells using laser scanning confocal microscopy. Representative micrographs shown are Z-series maximum intensity projections. (A) Expression of HopW1-RFP and Lifeact-GFP F-actin marker in Nicotiana benthamiana leaves 36–40 h after co-transformation with Agrobacteria. Micrographs show localization of cytoplasmic mCherry (control, upper panel) and HopW1-RFP (lower panel) together with Lifeact-GFP. GFP/RFP fluorescence is shown in green/red, respectively. Dotted line shows cells expressing HopW1-RFP. Bar = 30 μm. (B) Arabidopsis Col protoplasts from a transgenic line that expresses Lifeact-GFP [26] were transfected with HopW1-CFP (lower panel) or without DNA (control, upper panel). Micrographs show Lifeact-GFP and HopW1-CFP 15 h after transfection. GFP/CFP fluorescence is shown in green/magenta, respectively, and chloroplast (chl) autofluorescence in blue. These experiments were repeated twice with similar results. The actin cytoskeleton was not detectable in all cells in which HopW1-RFP/CFP was observed (at least 30 N. benthamiana cells and 40 Lifeact-GFP Arabidopsis protoplasts, respectively, with HopW1 signal were observed).

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to monitor endocytic trafficking [31] in Arabidopsis protoplasts. Over time (0.5 to 2 h after dye application), increased numbers of endocytic vesicles were stained with FM4-64 in wild-type protoplasts. In contrast, FM4-64 failed to label vesicles in protoplasts from transgenic Arabidopsis expressing dex-inducible HopW1 (Figure 6A and 6B). Endosomes labeled by FM4-64 were also significantly reduced in the LatB-treated versus control protoplasts (Figure 6B). Thus, HopW1 affects actin-dependent cell biological events when directly expressed in plant cells.

Infection Causes HopW1-Dependent Disruption of Endocytosis

We tested whether the delivery of HopW1 during P. syringae infection has a similar effect on endocytosis as HopW1 expressed directly in plant cells. To estimate when the inhibition of endocytosis might occur, we infected seedlings with PtoDC3000/HopW1 or PtoDC3000/vector, or treated seedlings with LatB as a positive control. We monitored endosomes stained with FM4-64 in Col cotyledons 1.5, 6, and 18 h after infection (Figure 7A and 7B). The numbers of endosomes per cell labeled with FM4-64 were highly reduced at 6 and 18 h after infection with PtoDC3000/HopW1 and similar to the effect of LatB treatment. In contrast, the number of endosomes was not different from mock treatment in early (1.5–6 h) PtoDC3000/vector infection, but was reduced by 50% at 18 h. These results indicate that HopW1 is mainly responsible for the inhibition of endocytosis during early stages of infection, but PtoDC3000 may have another factor(s) that also weakly affects endocytosis at the later times. The timing of inhibition of endocytosis by HopW1 is consistent with disruption of actin cytoskeleton during infection (Figure 2).

Discussion

HopW1’s virulence activity is strongly linked to its effect on actin and actin-dependent processes in susceptible Col Arabidopsis. Specifically, HopW1 co-purifies with actin from plants and can disrupt F-actin in vitro and decrease actin filament density during infection. Figure 8 shows a model for HopW1’s possible mode of action as a virulence factor. HopW1 inhibits actin-dependent cell biological processes in planta, such as endocytosis and the trafficking of certain proteins destined for ER and/or vacuoles. Surface receptors such as FLS2 or LeEix2 that recognize PAMPs and contribute to basal defense [32] are endocytosed upon activation [33,34,35]. Endocytosis of receptors requires an intact actin cytoskeleton [33], but whether endocytosis per se is critical for FLS2 signaling to limit bacterial growth is not clear. Endocytosis of the tomato receptor LeEix2 is important for its immune signaling output [35]. Trafficking to the ER, disrupted by HopW1, is necessary for replacement of endocytosed receptors at the plasma membrane [33,36] and secretion of antimicrobial factors [37]. Vacuole also has an established role in immunity [38].

HopW1 joins a growing list of pathogenic effectors from infectious bacteria that directly bind actin and/or regulate actin and thus actin-dependent processes [15,39,40,41,42,43]. However,
HopW1 is the first effector that we know of from a plant bacterial pathogen that directly targets actin. HopW1 can increase the growth of a \textit{P. syringae} isolate that is already relatively successful in growing on Arabidopsis. This implicates an actin-dependent process(es) as important for imparting an immune response to limit the growth of pathogenic bacteria.

Disruption of F-actin polymerization with LatB also increases susceptibility of the Col accession of Arabidopsis to \textit{Pto DC3000} ([12], this paper) confirming the role of actin cytoskeleton in defense. HopW1 is not part of the \textit{Pto DC3000} effector repertoire, and thus we expect that there may be yet additional effectors from \textit{Pto DC3000} and/or other \textit{P. syringae} strains that affect actin or actin-dependent processes.

Recently, a study of actin dynamics during infection of Arabidopsis by \textit{Pto DC3000} implicated effectors as causing increased F-actin bundling late in the infection as part of the virulence mechanism, although no specific effector was identified.
We did not notice any effect of HopW1 on F-actin bundling. An early response to PAMPs and infection is an increase in density of actin filaments [12,13], possibly to assist with higher demand for intracellular trafficking during defense. HopW1 counteracts this response by disrupting F-actin early during infection.

HopW1-C activity in vitro is specific for non-muscle (cytosolic) actin. Plant vegetative actin isoforms (expressed in all vegetative organs) are not more phylogenetically similar to non-muscle than muscle animal actins. However, they are functionally more related to cytosolic actins than to specialized muscle actins [44]. Cytosolic actin is essential for processes such as growth and intracellular trafficking in all eukaryotic cells. Our results confirm this functional similarity, recently reported by complementation of Arabidopsis vegetative actin mutants by human and protist cytosolic actins, but not by human muscle actins [44].

Figure 7. PtoDC3000/HopW1 infection inhibits endocytosis. (A) Examples of microscopic images of infected tissue in which endosomes are visualized using FM4-64. Cotyledons of Arabidopsis Col seedlings grown on MS plates were infected with PtoDC3000 carrying either empty vector (pME6012) or vector with the HopW1 gene at OD600 = 0.01. 100 μM LatB was used as an actin cytoskeleton-disrupting control. After infections and treatments for the indicated times, cotyledons were labeled for 1 h with FM4-64 and viewed. Arrows indicate some of the FM4-64-labeled endosomes. (B) Quantitation of the data in (A). Endosomes per cell were manually counted in at least 10 images per treatment, per time-point, from two or three biological repeats. Bars indicate SEM. Different letters indicate significantly different numbers of endosomes for given treatments, as determined by ANOVA/Tukey’s test (P<0.05). doi:10.1371/journal.ppat.1004232.g007
Interestingly, the actin-disrupting/actin binding portion of HopW1 (the C-terminal region) is also present in an effector found in some pathogenic E. coli strains [22]. A protein BLAST search indicates that the plant pathogen Acidovorax encodes a protein with similarity to the C-terminus of HopW1 (e.g. GeneBank accession no. YP 969911). These homologs may also disrupt actin as part of their virulence mechanisms. HopW1 does not have recognizable features corresponding to any known actin depolymerizing or severing factors. However, its localization pattern in patches resembled that of actin-binding proteins involved in F-actin organization at the membrane: class I formins, such as AtFH4 [45] and NET proteins [46]. Future structural analysis may shed light whether HopW1 is an example of a structural mimic of a known activity or whether it disrupts F-actin by a wholly novel mechanism.

Materials and Methods

Bacterial Strains and Plasmids
Bacterial strain and plasmids used in this study are listed in Tables S1, S2.

Bacterial Growth Assays In planta
Bacteria and/or 10 μM LatB were inoculated into leaves of 3-week old Arabidopsis thaliana Col accession grown in soil for bacterial growth analyses, which were done as described in Text S1.

Immunoprecipitation, Western Blot and LC-MS/MS analysis
HopW1-HA was immunoprecipitated with anti-HA matrix (Roche) from transgenic Arabidopsis or transiently transformed N. benthamiana as described Text S1. LC-MS/MS protein identification after trypsin digestion of protein bands was performed at Chicago Biomedical Consortium as described [47]; data was searched against the NCBI database using Mascot and validated with Scaffold 2 (Proteome Software Inc.)

Evaluation of F-actin Arrays in Cotyledons after Infection
To quantitatively evaluate actin filament populations, we calculated F-actin density in the cell as the percent occupancy of Lifeact-GFP signals based on the captured confocal microscope images. Transgenic Col/Lifeact-GFP [26] cotyledons were infected with PtoDC3000/empty vector or PtoDC3000/HopW1 at OD600 = 0.01 and 100 μM LatB was used as an actin cytoskeleton-disrupting control. Laser-scanning confocal microscopy was used to visualize Lifeact-GFP [see Text S1 for details] in infected epidermal cells. GFP fluorescence from Z-series maximum-intensity projections of 32 optical sections (0.5 μm each) was separated from background by minimal threshold to include all F-actin signals. Images were analyzed by Image J software (http://rsb.info.nih.gov/ij) as described [25]. Gaussian blur and high-band pass filter (1–5 pixels) were applied as described in [12,25]. Actin filament density was designated as a percentage of total pixel numbers of F-actin (as defined by Lifeact-GFP) per total number of pixels in the photograph [12,25]. Picture regions without stomata were used for F-actin density calculation.

Protoplast Isolation and Transient Transformation
Arabidopsis protoplasts isolation, polyethylene glycol (PEG)-mediated transformation [48,49] and microscopy were done as described in Text S1. Protoplasts from at least two independent transgenic dex:HopW1 lines were used with similar results.

Agrobacterium tumefaciens GV3101 strain harboring pMDG43-Lifeact (F-actin marker) and 35S-mCherry (control) or pGWB454-HopW1 (HopW1-RFP) were co-infiltrated into N. benthamiana leaves. 16–40 h after Agro-infiltration, leaves were analyzed by confocal microscopy. At least 30 cells with HopW1-CFP signal were observed in 2 experiments 24–40 h after transformation.

Monitoring Protein Trafficking in the Presence of HopW1
Constructs for in vivo organelle targeting reporter proteins (AAALF-GFP and SPO-GFP, kindly provided by Dr. Inhwan Hwang in Pohang University of Science and Technology, South Korea) were transfected into protoplasts derived from Arabidopsis dex:HopW1 and control non-transgenic plants, and incubated in W5 buffer with 0.2 μM dexamethasone to express HopW1. Protoplasts were monitored using confocal fluorescence microscopy.
(described in Text S1) at the various times (12 h, 24 h, and 48 h) after transfection/dex treatment. Based on the digital images of transformed protoplasts, we categorized GFP patterns by the distributions of reporters as a vacuolar, an ER, and a punctate pattern, and then we counted and scored the distribution patterns of >100 protoplasts.

Endocytosis Inhibition Assay in Protoplasts and Cotyledons

Protoplasts prepared from Arabidopsis expressing dex:hopW1 or non-transgenic controls were incubated overnight with 0.2 μM dexamethasone and then stained with 6.4 μM FM4-64 (Invitrogen, Eugene, OR) for 5 min and washed with W5 buffer. The protoplasts were then incubated for various times (up to 2 h) in W5 buffer at room temperature prior to fluorescence microscopy as described in Text S1.

To study endocytosis after infection, 6- to 8-d old Arabidopsis Col grown on MS agar plates were inoculated by placing a drop of PstDC3000/HopW1 or PstDC3000/vector cultures at OD600 of 0.01 in 10 mM MgSO4 or 100 μM LatB on each seedling, and incubated in a sterile hood for 30 min to allow the excess liquid to be absorbed by the seedling or evaporate. Seedlings were returned to the growth chamber for various times (up to 24 h). Harvested cotyledons infiltrated with 8.2 μM FM4-64 (Invitrogen, Eugene, OR) using vacuum for 2 min, were incubated 1 h prior to microscopic analyses. Endosomes were manually counted in at least 10 plant cells per treatment per time point.

Non-muscle F-actin Disruption Assays

F-actin disruption assays followed manufacturer’s protocol (Cytoskeleton, #BK013, USA) for sedimentation assays or fluorescence microscopy as described [50,51] in the presence of purified recombinant HopW1-C (Text S1). Non-muscle actin was polymerized to filaments in 10 mM Tris pH 7.0, 1 mM ATP, 50 mM KCl, 1 mM EGTA, 0.2 mM CaCl2 and 2 mM MgCl2 for 1 h at room temperature. 10 μM preassembled F-actin was incubated with 0.5 mM dex:HopW1-C or controls (BSA or E. coli extract) for 30 min at room temperature and centrifuged at 150,000×g for 1.5 h. Pellet (P) and supernatant (S) fractions were separated by SDS-PAGE.

To examine F-actin disruption by fluorescence microscopy, 5 μM preassembled non-muscle F-actin was incubated with different amounts of HopW1-C or BSA for 1 h at room temperature and stained for 5 min with 1 μM TRITC-Phalloidin (Fluka Biochemika, Switzerland). Reactions were terminated by a 250-fold dilution in fluorescence buffer (50 mM KCl, 1 mM MgCl2, 100 mM DTT, 20 μg/ml catalase, 100 μg/ml glucose oxidase, 3 mg/ml glucose, 0.5% methylcellulose, and 10 mM imidazole, pH 7.0) and absorbed to coverslips coated with 0.05 μg/ml poly-L-lysine.

Fluorescence images were collected with a cooled CCD camera (Orca-ER, Hamamatsu) on an Olympus IX-81 microscope. The lengths of at least 100 filaments per treatment were quantified using ImageJ software (http://rsb.info.nih.gov/ij).

Supporting Information

Figure S1 C-terminal domain of HopW1 forms complexes with actin in plants. N. benthamiana was transiently transformed using Agrobacteria carrying HopW1 domains or full length tagged with HA. Complexes were immunoprecipitated with anti-HA agarose from dexamethasone-treated leaves and actin was detected by immunoblotting. *, not transformed control; N, HopW1-N-HA (dex:HopW1<sub>18</sub>–761-HA); C, HopW1-C-HA (dex:HopW1<sub>18</sub>–417-HA); W1, full length HopW1-HA (dex:HopW1<sub>1</sub>–774-HA). Asterisks (*) mark bands corresponding to monomeric HopW1-HA variants (HopW1 and HopW1-C are also detected in larger bands that may be dimers). Input was 3% of extract used for each IP. This experiment was repeated 3 times with similar results. Note that accumulation of HopW1-C is lower than other variants.

Figure S2 HopW1 did not disrupt muscle F-actin. Visualization of muscle F-actin. 0.5 μM of BSA (i), or 0.5 μM of HopW1-C (ii) was incubated with pre-assembled muscle F-actin (from chicken breast) for 1 h. Actin filaments were stained with TRITC-phalloidin and observed by epifluorescence microscopy. At least 100 actin filaments were measured from each sample and filament lengths were quantified (right panel). This experiment was repeated twice with similar results.

Figure S3 Phenotypic effects of LatB on AALP:GFP and SPO:GFP localization. (A) Example of microscopic analysis of the effect of LatB on AALP:GFP and SPO:GFP localization. Protoplasts from wild-type plants were transfected with AALP:GFP or SPO:GFP and incubated in 10 μM of LatB. Localization of AALP:GFP and SPO:GFP was examined using confocal fluorescence microscopy per time point, in two biological repeats. In the presence of LatB, the distribution patterns of the AALP:GFP and SPO:GFP showed similar punctate fluorescence patterns similar to those caused by HopW1. (B) Quantitation of the LatB-altered distribution patterns of AALP:GFP and SPO:GFP in Arabidopsis. Protoplasts were counted based on the distribution patterns in the presence and absence of LatB 12 h, 24 h, and 48 h after transfection from two biological repeats. Bars indicate SEM, χ² tests indicated that the distributions were significantly different between the wild type and LatB treatment at each time point for each marker protein fusion (P<0.0001, n≥30).

Table S1 Bacterial strains.

Table S2 Plasmids.

Text S1 Contains supporting materials and methods and supporting references.

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Author Contributions

Conceived and designed the experiments: JJ YK NMC YL DRK JTG. Performed the experiments: YK JF YMC YL. Analyzed the data: JJ YK NMC WYL. Contributed reagents/materials/analysis tools: JJ YK NMC. Wrote the paper: JJ JTG YMC YL DRK.

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