

Plant pathogenic bacteria target the actin microfilament network involved in the trafficking of disease defense components

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Cells of infected organisms transport disease defense-related molecules along actin filaments to deliver them to their sites of action to combat the pathogen. To accommodate higher demand for intracellular traffic, plant F-actin density increases transiently during infection or treatment of Arabidopsis with pathogen-associated molecules. Many animal and plant pathogens interfere with actin polymerization and depolymerization to avoid immune responses. *Pseudomonas syringae*, a plant extracellular pathogen, injects HopW1 effector into host cells to disrupt the actin cytoskeleton and reduce vesicle movement in order to elude defense responses. In some Arabidopsis accessions, however, HopW1 is recognized and causes resistance via an actin-independent mechanism. HopW1 targets isoform 7 of vegetative actin (ACT7) that is regulated by phytohormones and environmental factors. We hypothesize that dynamic changes of ACT7 filaments are involved in plant immunity.

Actin is Targeted by Pathogen Effectors to Disrupt Intracellular Traffic and Immunity

Many pathogens target actin to remodel the host cytoskeleton. Most often actin targeting is observed in intracellular pathogens to enable their entry to the cell. Animal/human pathogens such as Salmonella or plant fungal and oomycete pathogens induce actin filament rearrangement leading to microbial uptake or penetration of a host cell by pathogen hyphae^{1–4}. Similarly, enteropathogenic *E. coli*, although extracellular, rearranges the host cytoskeleton to build pedestals to which it attaches and multiplies.⁵ However, the cytoskeleton is also a network used to deliver antimicrobial agents to sites of infection e.g. secrete antimicrobial compounds, reinforce plant cell wall by callose deposition or deliver pathogen-associated molecular pattern (PAMP) receptors and other elements of the immune response machinery to the plasma membrane.⁶ Some effectors from pathogenic bacteria interfere with these defense processes: for example VipA of *Legionella* or YopE of *Yersinia* target actin in animal cells to disrupt intracellular trafficking to avoid the immune response and phagocytosis.^{7–11} Inhibition of endosomal trafficking and phagocytosis is achieved by different ways of interference with actin dynamics: VipA⁷ is an actin nucleator, whereas YopE^{9,10} has Rho GAP activity that disrupts actin filaments.

HopW1 of *Pseudomonas Syringae* Disrupts Actin in Plants

HopW1 of *P. syringae* PmaES2643 is the first bacterial effector shown to target actin in plants.¹² *P. syringae* is an extracellular pathogen that injects effectors into plant cells via a type III secretion system in order to suppress immune responses and create an environment supporting bacterial growth. HopW1 binds and solubilizes F-actin and disrupts actin-dependent trafficking, such as endocytosis and vacuole targeting in infected plants. This activity of HopW1 leads to increased virulence of *P. syringae* in the Col accession of Arabidopsis. We showed that the C-terminal domain of HopW1 disrupts cytosolic actin filaments *in vitro* and the full-length protein expressed in plants cells disorganizes the cytoskeleton to such a degree that actin filaments visualized by Lifeact-GFP (a protein that binds actin fused to GFP to allow fluorescence imaging in living cells) cannot be detected.¹²

HopW1 has no similarity to any proteins of known functions but its N-terminal domain is also found in the N-terminal region of HopAE from *P. syringae* pv. *syringae* and its C-terminal domain is present in a predicted effector (EspW) from pathogenic *E. coli*.¹³ It is possible that EspW is another *E. coli* effector that targets actin and may have filament-disrupting activity similar to HopW1.

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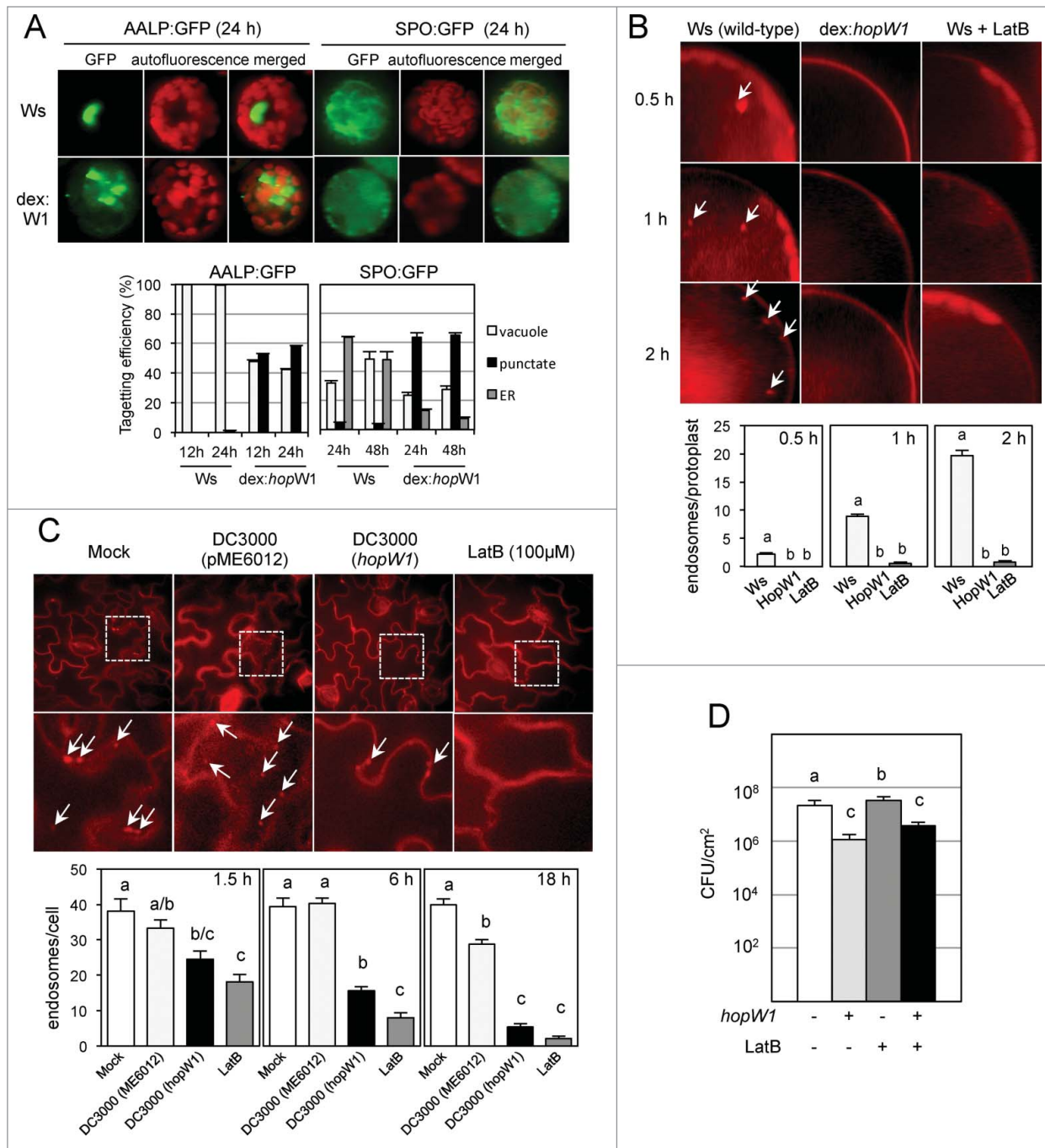


Figure 1. For figure legend, see page 151.

Dynamic Changes of the Actin Cytoskeleton are Necessary for Plant Defense

Cytoskeleton changes have been implicated in immune responses in plants. Actin depolymerization factor 4 (ADF4) is necessary for transcriptional activation of the resistance gene RPS5 and MAP kinase signaling in response to the *P. syringae* effector AvrPphB.^{14,15} During infection with virulent *P. syringae* DC3000

or treatment with PAMPs, transient increase of F-actin density was observed, probably to satisfy the requirement for increased intracellular traffic.^{16,17} Responses to one of PAMPs, a fragment of bacterial elongation factor (efl26), were linked to inhibition of ADF4.¹⁷ A recent publication reported that pathogen infection also increases the numbers of multivesicular bodies and exocyst vesicles related to relocation of defense compounds and that endosomal trafficking is essential for basal defense against *P. syringae*.¹⁸

Actin depolymerization by 10 μM latrunculin B (LatB) induces plant susceptibility to bacterial infection^{12,16}, however treatment with 200 nM LatB was shown to induce salicylic acid (SA)-dependent gene transcription (pathogen growth in plants treated with low concentrations of LatB was not tested¹⁹). SA is a major defense regulator in plants and induces transcription of genes correlated with resistance. High concentrations of SA (1 mM) caused F-actin disruption.¹⁹

Effectors and Toxins that Target Actin may also Induce Immune Responses

Consistent with induced susceptibility, HopW1 that disrupts F-actin in plant cells is a virulence factor during infection of susceptible *Arabidopsis* (Col accession). However in the *Ws* accession of *Arabidopsis*, HopW1 is recognized and induces defense responses via WIN2 and WIN3 proteins that interact with HopW1. Defense activation in *Ws* and WIN protein binding were attributed to the C-terminal domain of HopW1,²⁰ the same domain that binds and disrupts F-actin.¹²

Several effectors or toxins of animal pathogens that target actin also induce immune responses (reviewed in²¹) and actin depolymerization was linked to NF κ B activation.²²

Recognition of HopW1 in a Resistant *Arabidopsis* Accession is not Related to Actin Disruption

The defense-inducing activity (avirulence) of many plant effectors results from detection of perturbation of their targets, therefore it was possible that disruption of F-actin could be monitored (guarded) in *Ws* and induce resistance. We tested if HopW1 interfered with actin-dependent intracellular trafficking in *Ws*, similarly to what we found in the susceptible Col

accession. Effects of expression of HopW1 in *Ws* protoplasts (Fig. 1) were the same as in Col or as treatment with the actin-depolymerizing agent LatB¹²: HopW1 reduced proper targeting of marker proteins to the ER and vacuole (Fig. 1A) and inhibited endocytosis (Fig. 1B). During infection with *Pto*DC3000 expressing HopW1, endocytosis was also reduced in *Ws* in comparison to infection with the *Pto* strain carrying empty vector (Fig. 1C) or mock-treated control. The effect of HopW1 on actin-dependent processes in *Ws* was similar to the effect of LatB treatment. However F-actin depolymerization by LatB slightly increased the growth of virulent strain *Pto*DC3000 in *Ws* (Fig. 1D), similarly as in Col, while HopW1 expression reduced *Pto* growth in *Ws*. Therefore recognition and the avirulent effect of HopW1 is not related to F-actin disruption, and actin depolymerization seems to increase susceptibility to *P. syringae* in different *Arabidopsis* accessions.

Plants have Multiple Actin Isoforms

In our previous study,¹² we found that the C-terminal domain of HopW1 disrupts animal cytoplasmic (non-muscle) F-actin, but has no activity toward muscle actin. Plants have multiple actin isoforms, e.g., *Arabidopsis* has at least 8 functional actin genes (plus 2 pseudogenes) divided into 2 major functional groups: vegetative (expressed in all vegetative tissues: *ACT* 2, 8 and 7) and reproductive (expressed in reproductive organs and meristems: *ACT* 1, 3, 4, 12 and 11) (reviewed in²³). Animal cytoplasmic and muscle actins or plant vegetative and reproductive actins are not interchangeable i.e. animal cytoplasmic actin mutant is not rescued by muscle actin and plant vegetative actin mutant is not fully rescued by reproductive actin. Plant actins are not more phylogenetically related to animal cytoplasmic than muscle actins however, vegetative actins seem to be functionally related to animal cytosolic actins and ancestral actins of unicellular organisms. Vegetative plant actin (*act7/8* mutant in

Figure 1 (See previous page). Effects of HopW1 on actin dependent processes and bacterial growth in *Arabidopsis* *Ws*. **(A) HopW1 inhibits vacuolar and ER trafficking.** Wild-type and *dex:hopW1* protoplasts were transfected with AALP:GFP or SPO:GFP, incubated in 0.2 μM of dexamethasone for indicated times and imaged by confocal microscopy. AALP:GFP was targeted to the central vacuole and SPO:GFP localized to the ER and vacuole in wild-type protoplasts (upper row), as previously documented for Col.¹² In the presence of HopW1, many protoplasts transfected with the AALP:GFP and SPO:GFP showed notable punctate patterns. Localization patterns were quantified from at least 100 images, see¹² for comparison with Col protoplasts and the effect of LatB. Bars indicate SEM. χ^2 tests indicated that the distributions were significantly different between the wild-type and *dex:hopW1* at each time point ($P < 0.0001$, $n \geq 100$ per genotype/fusion construct). **(B) HopW1 inhibits endocytosis in protoplasts.** Representative microscopic images show effects of HopW1 and LatB on endocytic vesicle formation. Wild-type and *dex:hopW1* protoplasts were treated with 0.2 μM dexamethasone, stained with FM4-64 and visualized by confocal microscopy. Ten μM LatB was used to disrupt the actin cytoskeleton. After over-night incubation with dexamethasone and/or LatB, protoplasts were labeled with FM4-64 and viewed after 0.5, 1 and 2 h. Arrows point to some of the FM4-64-stained endosomes. Endosomes were quantified in at least 20 protoplasts per treatment, per time-point in 3 independent experiments. Bars indicate SEM, and letters indicate significantly different number of endosomes ($P < 0.0001$, ANOVA/Neuman-Keuls test). **(C) HopW1 inhibits endocytosis during infection.** Cotyledons of *Arabidopsis* *Ws* seedlings grown on MS plates were infected with *Pto*DC3000 carrying either empty vector (pME6012) or vector with the HopW1 gene at $\text{OD}_{600} = 0.01$. 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. Examples of microscopic images of tissue 6h after infection are shown. Lower row is magnification of fragments from pictures in upper row. Arrows indicate some of the FM4-64-labeled endosomes. Endosomes per cell were manually counted in at least 8 images per treatment, per timepoint, from 2 biological repeats. Bars indicate SEM and letters indicate significantly different numbers of endosomes for given treatments ($P < 0.05$, ANOVA/Neuman-Keuls test). **(D) Disruption of actin cytoskeleton does not induce resistance in *Arabidopsis* *Ws*.** Growth of *Pto*DC3000/vector or *Pto*DC3000/HopW1 ($\text{OD}_{600} = 0.0001$) 3 d post-inoculation of *Ws* accessions was monitored in the presence or absence of 10 μM LatB. Different letters indicate significantly different growth (ANOVA/Neuman-Keuls test, $P < 0.05$). LatB did not trigger resistance in *Ws*, whereas HopW1 did, suggesting that the mechanism of resistance does not involve disruption of the actin cytoskeleton. Average of all results with SEM from 3 independent experiments ($n=9$) is shown. All experiments were repeated 3 times with similar results. Detailed methods are described in ref.¹²

Table 1. Actin peptides identified in HopW1 complex immunoprecipitated from *N. benthamiana* by LC-MS/MS and search of whole Genbank protein sequences. Identity to Arabidopsis actin isoforms is indicated (actin isoforms are annotated in Arabidopsis and some other species but not in *N. benthamiana*). *This peptide has 100% identity to Glycyrrhiza and Mimosa sp. actin.

	Actin peptides identified in HopW1 complex	Identity to At peptide	At actin isoforms with matching peptides
1	AVFPSIVGRPR	100%	actin 4,7,3,12,11
2	DAYVGDEAQS	100%	actin 2,4,7,3,12,11,8
3	DAYVGDEAQS	100%	actin 2,4,7,3,12,11,8
4	DLYGNIVLSGGSTMFPGIADR	100%	actin 7
5	GEYDESGSIVHR	100%	actin 4,7,3,12,11
6	GYMFTTSAER	90% *	actin 2,7,8,11
7	HTGVMVGMGQK	100%	actin 4,7,3,12,11
8	IWHHTFYNELR	100%	actin 2,4,7,3,12,11,8
9	LAYVALDYEQELETAK	100%	actin 7
10	NYELPDGQVITIGAER	100%	actin 2,7,8
11	AGFAGDDAPR	100%	actin 2,4,7,3,12,11,8
12	TTGIVLDSGDVSHTVPIYEGYALPHAILR	100%	actin 4,7,3,12,11
13	VAPEHPVLLTEAPLNPK	100%	actin 4,7,12,11

Arabidopsis) can be complemented by cytoplasmic (non-muscle) human/animal actins and protist actins, but not by muscle actins.²⁴

HopW1 Targets Vegetative Actin 7 that Responds to Hormones and Environment

Most of the actin peptides found in a complex with HopW1 in *N. benthamiana*¹² match multiple actin isoforms, however 2 peptides are specific for a vegetative actin 7 (ACT7) and ACT7 is the only isoform that all other peptides also match (Table 1). Both HopW1 activity on animal cytosolic F-actin and proteomics analysis of HopW1 complexes *in planta* suggest that HopW1 has specificity toward vegetative actin(s) in host cells. Although the identity of actin isoforms involved in plant response to pathogens was not determined, these are very likely vegetative actins involved in vesicle trafficking and transport in the cells of

infected tissue. ACT7 targeted by HopW1 seems to be the only plant actin found responsive to external stimuli and phytohormones. ACT7 expression is induced by auxin, light and wounding and suppressed by abscisic acid.^{25,26} It would be intriguing to know if ACT7 dynamics are specifically affected by infection and if this actin isoform is responsive to salicylic acid or other hormones involved in defenses.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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