Structure of a pathogen effector reveals the enzymatic mechanism of a novel acetyltransferase family

Zhi-Min Zhang1,8, Ka-Wai Ma2,8, Shuguang Yuan3, Youfu Luo4, Shushu Jiang2,7, Eva Hawara2, Songqin Pan5, Wenbo Ma2,5,6 & Jikui Song1

Effectors secreted by the type III secretion system are essential for bacterial pathogenesis. Members of the Yersinia outer-protein J (YopJ) family of effectors found in diverse plant and animal pathogens depend on a protease-like catalytic triad to acetylate host proteins and produce virulence. However, the structural basis for this noncanonical acetyltransferase activity remains unknown. Here, we report the crystal structures of the YopJ effector HopZ1a, produced by the phytopathogen Pseudomonas syringae, in complex with the eukaryote-specific cofactor inositol hexakisphosphate (IP6) and/or coenzyme A (CoA). Structural, computational and functional characterizations reveal a catalytic core with a fold resembling that of ubiquitin-like cysteine proteases and an acetyl-CoA-binding pocket formed after IP6-induced structural rearrangements. Modeling-guided mutagenesis further identified key IP6-interacting residues of Salmonella effector AvrA that are required for acetylating its substrate. Our study reveals the structural basis of a novel class of acetyltransferases and the conserved allosteric regulation of YopJ effectors by IP6.

Gram-negative bacterial pathogens use the type III secretion system (T3SS) to inject virulence proteins into plant and animal cells1. These type III–secreted effectors (T3SEs) are essential for pathogenesis and manipulate specific physiological processes and immune signaling pathways2. T3SE repertoires are highly variable in different pathogen species, owing to rapid adaptation to their specific host interactions. An exception is YopJ, which is the most widely distributed T3SE family and includes members produced by both plant and animal pathogens3. This conservation suggests the important virulence functions of this effector family. To date, 11 YopJ family effectors produced by the human pathogens Salmonella enterica, Yersinia spp. and Vibrio parahaemolyticus, and the plant pathogens Ralstonia solanacearum, P. syringae and Xanthomonas campestris have been found to contribute to pathogenesis by suppressing host immunity.

Although they bear a conserved histidine-glutamate-cysteine or histidine-aspartate–cysteine catalytic triad identical to that of the CE clan of cysteine proteases4, many YopJ family members have acetyltransferase activities5–14. Interestingly, there is no sequence similarity between YopJ effectors and the other acetyltransferases, thus indicating that this activity is noncanonical. Indeed, the activities of YopJ effectors are uniquely stimulated by the eukaryote-specific ligand IP6 (refs. 14–16); furthermore, they modify specific serine and threonine residues of their host targets, in addition to the canonical lysine residues.

Acetylation is an important post-translational modification that affects various aspects of protein function by influencing stability, subcellular localization and enzymatic activity. The best-studied example comes from histones, in which acetylation of specific lysine residues determines chromatin structures and gene expression17. Investigation of the host targets of YopJ effectors has revealed diverse substrates that are inactivated through acetylation. Importantly, the acetylated substrate residues are directly linked to the substrates’ functions in immunity. For example, the Yersinia effector YopJ and the Salmonella effector AvrA acetylate the threonine, serine and/or lysine residues in the activation loop of specific mitogen-activated protein kinase kinases (MKKs); as such, these effectors block phosphorylation and inhibit immune signaling15,16. PopP2, produced by the plant pathogen R. solanacearum, modifies the WRKY DNA-binding domain, which is present in a class of defense-related transcription factors, and a specific PopP2-recognizing immune receptor11,12. Acetylation of the key lysine residue in the WRKY domain disrupts its interaction with DNA and manipulates defense-gene expression11,12. However, the biochemistry and the regulatory mechanism underlying this novel group of serine-threonine-lysine acetyltransferases remain largely unknown.

To determine the mechanistic basis of YopJ effector-mediated acetylation, we solved crystal structures of the P. syringae effector HopZ1a in complex with IP6 and/or CoA, a byproduct of the cofactor acetyl-CoA (AcCoA). These structural studies, together with computational and functional analyses, reveal the structural basis for the activity of this novel family of acetyltransferases and their conserved allosteric regulation by IP6.

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1Department of Biochemistry, University of California, Riverside, Riverside, California, USA. 2Department of Plant Pathology and Microbiology, University of California, Riverside, Riverside, California, USA. 3Laboratory of Physical Chemistry of Polymers and Membranes, École Polytechnique Fédérale de Lausanne, Lausanne, Switzerland. 4State Key Laboratory of Biotherapy, West China Hospital, Sichuan University, Chengdu, China. 5Center for Plant Cell Biology, University of California, Riverside, Riverside, California, USA. 6Institute of Integrative Genome Biology, University of California, Riverside, Riverside, California, USA. 7Present address: Sainsbury Laboratory, Norwich, UK. 8These authors contributed equally to this work. Correspondence should be addressed to W.M. (wenbo.ma@ucr.edu) or J.S. (jikui.song@ucr.edu).

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RESULTS

Crystal structures of HopZ1a in complex with IP6 and/or CoA

Because crystallization of wild-type HopZ1a has not been successful despite numerous trials, we generated two mutants in which either eight or ten putative surface residues were replaced with tyrosine or alanine. The constructs also contained a deletion of the first 28 amino acids, which are predicted to be disordered (Fig. 1a). These constructs eventually permitted crystallization and structural analyses of HopZ1a–IP6 at 2.0-Å resolution and HopZ1a–IP6–CoA at 3.4-Å resolution (Fig. 1, Supplementary Fig. 1 and Table 1).

HopZ1a comprises 14 α-helices and 8 β-strands, which fold into two closely packed domains (Fig. 1b,c and Supplementary Fig. 1a): a catalytic domain assuming a ubiquitin-like protease (ULP) fold19 and a regulatory domain formed by flanking sequences. The catalytic domain consists of a six-stranded β-sheet sandwiched by αC, αF, αG and αI from one side and αD, αE, αH and αI from the other side (Fig. 1b,c). The catalytic triad, composed of H150, E170 and C216, is situated at one end of the β-sheet, and H150 bridges E170 and C216 through hydrogen bonds (Fig. 1d and Supplementary Fig. 1b,c). The side chain of H150 also assumes an alternative conformation in which it points outward because of the crystal-packaging effect (Supplementary Fig. 1c).

The H150-E170-C216 catalytic triad is similar to that of the CA family of cysteine proteases (for example, ULPs) in sequence identity and topology (Fig. 1d). A DALI structural homology search20 revealed that HopZ1a shares high similarity with ubiquitin-like protease 1 (ULP1)19, with a backbone r.m.s. deviation of 2.96 Å over 148 residues. However, HopZ1a does not show considerable homology with any reported acetyltransferases, such as the GCN5-related N-acetyltransferase (GNAT)21 or the MYST family of acetyltransferases22. Comparison with ULP1 also revealed that the small ubiquitin-like modifier (SUMO)-recognition site, including a surface groove guiding the C-terminal loop for proteolytic cleavage, is no longer present in HopZ1a (Fig. 1d) or other Yop family effectors (Supplementary Fig. 2). However, the region spanning from the αG helix to the β8 strand of HopZ1a appears to be a unique element of Yop family effectors (Fig. 1d and Supplementary Fig. 2). These observations explain how Yop family effectors have lost their SUMO protease activity and acquired new structural features conferring acetyltransferase activity, and they also support the hypothesis that Yop family effectors and ULP1-like cysteine proteases diverged from a common ancestor23. Importantly, these data suggest that HopZ1a may have adopted the strategy used by cysteine proteases for enzymatic catalysis, including the formation of an acetyl-enzyme intermediate and subsequent transfer of the acetyl group to the attacking nucleophile23.

HopZ1a shows predominant acetyltransferase activity on serine and threonine residues16. Inspection of its catalytic core revealed that the catalytic cystine C216 is situated at the base of a shallow groove that is surrounded by S87 and S213 (Supplementary Fig. 1b).

Such a shallow active site pocket may explain why serine and threonine, which have small side chains, are favorable targets for HopZ1a-mediated acetylation.

HopZ1a–IP6 interaction is required for HopZ1a function

The IPα molecule lies on a positively charged concave surface created by six α-helices spreading over the N-terminal (αA and αC), middle (αF) and C-terminal (αK, αM and αN) regions (Fig. 1b and Supplementary Fig. 1d,e); the surface is further closed by a long loop connecting αL and αM (loopαL–αM) (Fig. 1b). Association of IPα with HopZ1a is mediated by a network of direct or water-mediated hydrogen-bonding interactions involving αA (R49 and K53), αC (R106), αF (N222, K226 and K229), loopαL–αM (K289 and H290), loopαL–αM (S314 and H317), αM (R326) and αN (Q358 and R362) and the six phosphate moieties of IPα (Fig. 2a).

To confirm the biological importance of key IPα-interacting residues, we generated HopZ1a mutants in which the evolutionarily conserved K226, K289 and R362 were each replaced with a glutamate, and we measured their IPα-binding affinity by using isothermal titration calorimetry (ITC). Wild-type HopZ1a bound IPα with a Kd of 22.7 µM, whereas the K226E, K289E and R362E mutations all led to abolished IPα binding (Fig. 2b and Supplementary Table 1). Next, we performed in vitro acetyltransferase analysis by using the jasmonate ZIM-domain protein 10 (JAZ10), a known target of HopZ1a in Arabidopsis thaliana, as the substrate. IPα stimulated the acetyltransferase activity of HopZ1a toward JAZ10 approximately eight-fold (Fig. 2c). By contrast, none of the three IPα-binding-deficient mutants acetylated JAZ10, regardless of the presence of IPα—a result similar to that for the catalytic inactive C216A mutant (Fig. 2c).

Wild-type HopZ1a elicited programmed cell death, called the hypersensitive response (HR), in Arabidopsis eco. Col-0 (ref. 3). Consequently, bacteria producing wild-type HopZ1a are restricted for their growth in plant tissue, thus leading to a decreased bacterial titer. Furthermore, leakage of cellular electrolytes increases because of the loss of membrane integrity in inoculated tissue. Because the HR-triggering activity of HopZ1a is dependent on the enzymatic activity, the appearance...
**Table 1 Data collection and refinement statistics**

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One crystal was used for each structure. *Values in parentheses are for the highest-resolution shell.

CoA-interacting residues in HopZ1a

YopJ effectors use AcCoA as the acetyl-group donor. In the crystal structure of the HopZ1a–IP₆–CoA complex, we observed that the CoA molecule was embedded in a surface groove winding across the regulatory and catalytic domains (Fig. 1b and Supplementary Fig. 1f,g). In particular, the adenylyl moiety of CoA is inserted into a pocket lined by loopβ₅–α₅m (K211 and T212), loopα₆–α₇ (A292) and loopβ₇–β₈ (R334 and F348). Meanwhile, the 3’-hydroxyl, 3’-phosphate and 5’-phosphophosphate groups of CoA are hydrogen-bonded to β8 (Q347); αm (L294 and T295) and β7 (R331); and loopβ₅–α₅ (K211), respectively (Fig. 3a). Additional HopZ1a–CoA interactions include van der Waals contacts involving loopβ₂–β₅ (H150), loopβ₄–α₄ (A172–V173) and loopβ₅–α₅ (K211–S213) (Fig. 3a), which guide the extension of the pantetheine arm of CoA toward the catalytic site. Notably, structural comparison of HopZ1a–IP₆ and HopZ1a–IP₆–CoA revealed a similar conformation of HopZ1a, with a Cα r.m.s. deviation of 0.4 Å over 287 residues, thus indicating that binding of CoA to the IP₆-bound HopZ1a does not involve substantial conformational readjustment.

To evaluate the biological importance of AcCoA-binding, we mutated K211, T295 and F348, which the structural analysis suggested to have direct interactions with CoA. These residues are located on different regions around the binding pocket. Importantly, K211 and F348 are conserved among Yop family effectors. Although single or double mutations led to inconsiderable or moderate reduction of the acetyltransferase activity, the K211A T295A F348A triple mutant was no longer able to acylate JAZ10 (Fig. 3b). Consistently with this observation, the K211A T295A F348A mutant was unable to bind AcCoA (Supplementary Table 1) or trigger HR in Arabidopsis (Fig. 3c,d and Supplementary Fig. 3). These results verify the residues in the AcCoA-binding pocket and confirm that the interaction with AcCoA is important for the acetyltransferase activity of HopZ1a.

**IP₆ activates HopZ1a through an allosteric mechanism**

The IP₆- and CoA-binding sites are structurally distant from each other (Fig. 1b). We therefore examined the mechanism by which IP₆
stimulates the enzymatic activity of HopZ1a. We analyzed he effect of IP₆ on the conformational state of HopZ1a through NMR spectroscopy, CD spectroscopy and ITC (Fig. 4a and Supplementary Fig. 4). First, 1D 1H NMR spectral analysis revealed that the addition of IP₆ led to an increase in chemical-shift dispersion in the aliphatic and aromatic proton regions of wild-type or C216A-mutant HopZ1a, thus suggesting that HopZ1a underwent IP₆-induced structural ordering. The addition of IP₆ also led to slight NMR signal broadening, probably because of the chemical exchange between the apo form and IP₆-bound HopZ1a (Supplementary Fig. 4a,b). Consistently with this result, CD spectral analysis demonstrated that the addition of IP₆ also led to a slight but notable decrease in ellipticity at 208 nm and 222 nm, which was indicative of increased helical content (Supplementary Fig. 4a,b and Supplementary Table 2). By contrast, IP₆ did not induce considerable changes in the CD or 1D 1H NMR spectra of the IP₆-binding-deficient mutants (Supplementary Fig. 4c–e). Furthermore, ITC analysis indicated that HopZ1a bound AcCoA in the presence of IP₆, with a Kᵣ of 93.7 μM; interestingly, this interaction was undetectable in the absence of IP₆ (Fig. 4a). Together, these data suggest that IP₆ induces a conformational rearrangement of HopZ1a that subsequently stimulates the interaction of HopZ1a with AcCoA.

We further tested this possibility through limited proteolysis of HopZ1a with elastase. In the absence of IP₆, elastase cleaved HopZ1a into two sets of prominent products with molecular weights of ~8 and ~28 kDa, respectively (Fig. 4b); however, IP₆ substantially inhibited the proteolytic cleavage. In contrast, CoA did not appear to influence the proteolytic cleavage (Supplementary Fig. 5). N-terminal sequencing of the cleavage products by mass spectrometry identified three major cleavage sites at A277, D286 and N313 (Fig. 4c and Supplementary Table 3). Among them, A277 and D286 are located apart from the IP₆-binding pocket, thus suggesting that changes in their proteolytic susceptibility arise from an IP₆-induced conformational change of HopZ1a rather than a direct blockage effect by IP₆ binding. These data suggest that IP₆ binding transits the conformation of HopZ1a from a structurally less ordered state to a protease-resistant, more compact state that facilitates AcCoA binding.

To provide mechanistic insight into the IP₆-mediated activation of acetyltransferase activity, we performed molecular dynamics (MD) simulations for HopZ1a, HopZ1a–IP₆ and HopZ1a–IP₆–CoA. First, analyses of r.m.s. fluctuation (r.m.s.f.) (Fig. 4d) and B factors (Supplementary Fig. 6a) identified loop β₇–8 as the regions with the largest reduction of conformational fluctuation by IP₆ (Fig. 4d and Supplementary Fig. 6a); as described above, these two loops are constituents of the IP₆- and CoA-binding sites, respectively.

Figure 3 Identification of the AcCoA-binding pocket. (a) Close-up view of the HopZ1a-CoA interactions. (b) In vitro acetylation assay of wild-type or mutants of HopZ1a (top), with protein amount indicated by Coomassie blue staining (bottom). Uncropped blot images are shown in Supplementary Data Set 1. (c) Effects of AcCoA-binding-disrupting mutations on the HR-triggering ability of HopZ1a in Arabidopsis. The asterisk indicates a leaf with HR. Ratios indicate number of leaves with HR in WT over the indicated mutant. (d) Bacterial populations of PtoDC3000 expressing wild-type HopZ1a or the K211A T295A F348A mutant in Arabidopsis. * * * P < 0.01 by two tailed Student’s t test (n = 4 independent plants). Source data for the bar graph are available online. Experiments in b–d were repeated at least twice with consistent results.

Figure 4 IP₆-dependent AcCoA binding in YopJ family effectors. (a) ITC binding curves of HopZ1a with AcCoA in the presence or absence of IP₆. The Kᵣ for wild-type HopZ1a represents the mean value of two independent experiments. (b) SDS–PAGE analysis of elastase-mediated limited proteolysis of HopZ1a. (c) Mapping of the elastase-cleavage sites (red sticks) on the structure of HopZ1a. (d) R.m.s.f. of atomic fluctuations of HopZ1a in complex with IP₆ and/or CoA. The regions corresponding to loop β₇–8 and loop β₇–8 are shaded in blue and yellow, respectively. (e) MD simulation of HopZ1a after IP₆ binding. The vector length correlates with the domain-motion scale. (f) In vitro acetylation assay of AvrA on MKK4 (top), with protein amount indicated by Coomassie blue staining (bottom). This experiment was repeated at least twice with consistent results. Uncropped blot images are shown in Supplementary Data Set 1. (g) Working model illustrating the acetyltransferase activation of YopJ family effectors by IP₆.
Furthermore, two of the IP₆-dependent elastase cleavage sites, D286 and N313, are located within the spatially proximate region of loopαL–αM (Fig. 4c); this result is consistent with their IP₆-dependent proteolytic susceptibility (Supplementary Table 3). In addition, principal component analysis and normal mode analysis revealed concerted movements of HopZ1a after IP₆ binding (Fig. 4e and Supplementary Movie 1): loopαL–αM underwent a motion toward IP₆ (Fig. 4e), which translated into a motion of loopβ₇–β₈ through helices αL and αM, thereby narrowing the AcCoA-binding pocket (Fig. 4e). In agreement with these two analyses, the correlation network analyses of HopZ1a, HopZ1a–IP₆ and HopZ1a–IP₆–CoA showed that loopαL–αM consistently grouped into the same domain sector as helices αL and αM, which communicates directly with loopβ₇–β₈ in the IP₆-bound form (Supplementary Fig. 6b). As a result of these movements, IP₆ binding drives HopZ1a into a conformation that not only stabilizes interaction with IP₆ but also becomes poised for AcCoA binding (Fig. 4e). Together, these observations strongly support allosteric stabilization of the AcCoA-binding site by IP₆ binding.

A conserved regulatory mechanism within the YopJ family

Structure-based sequence analysis indicates high conservation of the IP₆- and the AcCoA-binding sites in YopJ family effectors (Supplementary Figs. 2 and 7a), thus suggesting that a similar regulatory mechanism is used for enzymatic activation throughout the family. To test this hypothesis, we examined the role of potential IP₆-binding residues in AvrA, a YopJ family effector produced by the animal pathogen S. enterica²¹. On the basis of the sequence alignment (23% identity and 40% similarity), we generated a structural model, which revealed a two-domain architecture similar to that of HopZ1a (Supplementary Fig. 7b). Guided by the model, we selected three putative IP₆-binding residues in AvrA for mutagenesis (K182E, K224E and R257E) and examined these mutants for acetyltransferase activity and conformational changes by measuring intrinsic tryptophan fluorescence. Consistently with previous observations¹⁵, the application of IP₆ resulted in a decrease in tryptophan fluorescence in wild-type AvrA, thus suggesting that IP₆ mediates a conformational change (Supplementary Fig. 7c). By contrast, none of the AvrA mutants showed noticeable changes in tryptophan fluorescence (Supplementary Fig. 7c), thus confirming their roles in IP₆ binding. In vitro acetyltransferase assays using human MKK4, one of the identified targets of AvrA²⁷, further indicated that mutations of these putative IP₆-binding residues led to decreased (K182E) or abolished (K224E and R257E) acetyltransferase activity even in the presence of IP₆ (Fig. 4f). These results together suggest a conserved IP₆-mediated allosteric regulation in YopJ family effectors (Fig. 4g).

DISCUSSION

Activation of bacterial virulence proteins by the eukaryote-specific ligand IP₆ has also been observed in the RTX toxin of Vibrio cholerae²⁴ and toxin A of Clostridium difficile²⁵. In these toxins, IP₆ binding introduces conformational distortions that facilitate the formation of substrate-binding pockets²⁴,²⁵. However, IP₆ serves as an allosteric switch that controls the association with the acetyl-group donor AcCoA in HopZ1a. These results suggest that YopJ effectors have developed a different regulatory strategy using the same eukaryotic ligand, so that the virulence function is enhanced inside the host cells.

Bacterial T3SSs directly influence pathogenesis². Structural insight into effector functions provides important guidance in the development of antimicrobial strategies and in drug design. Our structural analyses of the YopJ family effectors define a new family of acetyltransferases without sequence or structural homology to known acetyltransferases. These virulence proteins have adopted the catalytic core of ULP1-like proteases and have evolved acetyltransferase activity through the acquisition of a novel regulatory domain. Many effectors mimic eukaryotic enzymes to manipulate cellular processes in the host². To date, serine/threonine acetyltransferases have not been reported in eukaryotes. The structure of HopZ1a provides an exciting opportunity to identify endogenous enzymes that may potentially possess this activity, and it represents an important step toward the understanding of acetylation as a prevalent post-translational modification. If this structure is unique to the YopJ family effectors, new drugs might be designed to target this enzyme while having low toxicity toward endogenous host acetyltransferases.

METHODS

Methods and any associated references are available in the online version of the paper.

Accession codes. Coordinates and structure factors for the HopZ1a–IP₆ and HopZ1a–IP₆–CoA complexes have been deposited in the Protein Data Bank under accession codes PDB 5KL1 and PDB 5KLO, respectively.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

Z.-M.Z. determined the crystal structures of HopZ1a complexes and conducted ITC assays. K.-W.M. characterized NMR, CD and fluorescence spectra of HopZ1a or AvrA proteins. K.-W.M., S.J. and E.H. performed in vitro acetylation assays and in vivo functional analyses. S.V. performed computational analysis. Z.-M.Z. and Y.L. crystallized HopZ1a complexes. Z.-M.Z. and S.P. performed limited proteolysis and mass spectrometry analysis. W.M. and J.S. designed and organized the study. Z.-M.Z., K.-W.M., S.Y., W.M. and J.S. prepared the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Protein purification. DNA encoding HopZ1a was cloned into a modified pRSF-Duet vector preceded by an N-terminal HiiN-SUMO tag. Twenty-eight amino acid residues at the N terminus were removed, owing to their predicted structural disorder. This peptide is presumably related to type III–dependent translocation but not involved in virulence function. Eight or ten surface mutations (KR2A, KR3A, K119Y, E120Y, K298Y, Q299Y, E366A, E367A and/or Q210A and K211A) were introduced by site-directed mutagenesis to facilitate crystal-packing interactions. The plasmids carrying HopZ1a mutants were transformed into BL21 (DE3) RIL cells (Novagen). When the cell density reached an OD_{600} of 0.8, protein expression was induced by 0.1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) at 20 °C overnight. For SeMet labeling, cells were cultured in M9 minimum medium to mid-log phase and then induced for 16 h with the addition of 50 µg/L of each of the following amino acids: lysine, phenylalanine, threonine, isoleucine, leucine, valine and 1-selenomethionine. Cells were harvested, resuspended and lysed in buffer A containing 50 mM Tris–HCl, pH 8.0, 25 mM imidazole, 1 M NaCl, 0.5 mM DTT and 1 mM PMSF. Histidine-tagged HopZ1a proteins were purified with a nickel column and eluted with buffer A supplemented with 250 mM imidazole. The eluted protein was incubated with ubiquitin-like–protease 1 (ULP1) on ice to cleave the His_{6}-SUMO tag, and the tag-free proteins were further purified by hydrophobic interaction chromatography (Phenyl HP, GE Healthcare). Peak fractions were collected, concentrated and applied to Superdex 200 16/600 (GE Healthcare) preequilibrated with buffer B (25 mM Tris, pH 7.5, 200 mM NaCl, 2 mM DTT and 5% glycerol) for final purification. Purified HopZ1a was stored at −80 °C at a concentration of 25 mg/mL for future use.

Full-length wild-type and mutant AvrA genes codon optimized for expression in Escherichia coli were synthesized (Integrated DNA Technologies) and cloned into the pRSET-Duet vector. Recombinant His_{6}-SUMO-tagged AvrA and His-GST-MKK4 (Addgene plasmid 29579) were expressed in BL21 (DE3) RIL cells and purified with a nickel column as described above.

Crystallization and structure determination. HopZ1a with the ten–amino acid mutations was mixed with 10 mM IP{sub 6} and incubated on ice for 10 min to allow complex formation. The HopZ1a–IP{sub 6} crystals were grown with the hanging drop method. Crystals appeared overnight in a buffer containing 18–26% PEG 550 MME, 0.1 M sodium citrate, pH 5.2, and 200 mM ammonium acetate. Microseeding was performed to improve the quality of the crystals. To generate the crystals of the HopZ1a–IP{sub 6}–CoA complex, the HopZ1a mutant with eight of the ten mutations (without Q210A and K211A) was mixed with IP{sub 6} and CoA at a molar ratio of 0.9:10:2 and crystallized in a buffer containing 20% PEG 10000, 0.1 mM HEPES, pH 7.0, 200 mM ammonium acetate and 0.5 mM DTT. Microseeding was performed to improve the quality of the crystals. The raw data were processed with Proteome Discoverer version 2.1 (Thermo Scientific) to generate mgf files that were used in a Mascot search (version 2.5) against a database consisting of HopZ1a, the entire E. coli proteome and common contaminant proteins such as human keratins (Matrix Science).

Mascot search parameters allowed various modifications including N-terminal dimethylation, K dimethylation, oxidation (M), N-terminal acetylation, Glu–pyro-Glu (N-terminal Q), Glu–pyro-Glu (N-terminal E) and no enzyme specified. Identified HopZ1a peptides with scores above significance (P < 0.05) are reported.

In vitro acetylation assays. In vitro acetylation assays were carried out to examine the acetyltransferase activity of HopZ1a and AvrA, as previously described. 4 µg of HopZ1a or 2 µg of AvrA was incubated with [1{sup 4}C]acyetyl-CoA (55 µCi/µmol) in 25 µL reactions (50 mM HEPES, pH 8.0, 10% glycerol, 1 mM DTT, 1 mM PMSF and 10 mM sodium butyrate) at room temperature for 3 h for HopZ1a and at 37 °C for 1 h for AvrA. 20 µg of MBP-JAZ10-His and 18 µg of His-GST-MKK4 were used as the substrates of HopZ1a and AvrA, respectively. Unless otherwise indicated, IP{sub 6} and AcCoA were added at a protein/IP{sub 6}/AcCoA molar ratio of 2:15:15 for HopZ1a and 1:8:25 for AvrA.

P. syringae infection assays. DNA encoding wild-type and mutant HopZ1a carrying a C-terminal HA tag were cloned into the vector pUCP20tk. The plasmids were transformed into P. syringae pv. tomato strain DC3000 (PtoDC3000) and an effector-less D28E mutant (PtoD28E). The P. syringae strain PtoDC3000 has been maintained in the MA laboratory. The strain PtoD28E was kindly provided by A. Collmer at Cornell University.

P. syringae strains were grown on King’s B agar at 30 °C overnight. The leaves of five-week-old A. thaliana plants (eco. Col-0) were infiltrated with bacterial suspensions of PtoDC3000 at an OD_{600} of 0.0001 (approximately 1 x 10^{8} cfu/mL) in 10 mM MgSO_{4}. The inoculated plants were transferred to a growth chamber (22 °C and 16 h/8 h light/dark regime, 90% humidity) for 3 d, and the bacterial populations were determined as colony-forming units (cfu) per square centimeter, as previously described.

To test the HR-triggering activity, leaves of five-week-old Arabidopsis plants (eco. Col-0) were infiltrated with bacterial suspensions of PtoD28E at an OD_{600} of 0.1. The inoculated plants were transferred to the growth chamber, and the cell-death symptoms were monitored at 20 h after inoculation. Ten leaves were tested in each treatment and yielded similar results. For the electrolyte leakage assay, four leaf discs of 1 cm² were soaked in ddH₂O for 20 min and then transferred into a tube containing 3 ml of ddH₂O. The conductivity of the solution was measured with a Connet 1 conductivity meter (Hanna Instruments) at room temperature. At least three replicates were measured for each treatment, and the data are presented as the average readings ± s.d.

Wild-type and mutant HopZ1a, tagged with HA, were expressed under the natural promoter in PtoD28E. Proteins were induced in M63 minimal medium containing 1% fructose overnight at room temperature. Induced HopZ1a proteins in the bacterial cells were detected by immunoblotting with an anti-HA antibody (Roche Diagnostics, cat. no. 1186743001). Validation of this antibody is provided in the manufacturer’s website.
Far-UV circular dichroism (CD). Purified wild-type and mutant HopZ1a proteins (20 mM sodium phosphate, pH 7.5, and 150 mM NaCl) were diluted to a final concentration of 1.88 mg/mL (approximately 50 µM). IP6 was added at a protein/IP6 ratio of 1:1, 1:2, 1:5. CD spectra of 50 µL sample in the far-UV region (190–260 nm) were collected in 0.1-mm path-length cells (Hellma) with a J-815 spectrophotometer (Jasco). Each spectrum was scanned continuously with a 1-nm bandwidth, with an accumulation of eight times to lower the noise level. This experiment was performed twice with independent batches of purified proteins and yielded similar results. To estimate the changes in the secondary structure of HopZ1a with the addition of IP6, CD spectra in the range of 200–250 nm were analyzed with Beta Structure Selection (BESTSEL)33.

Tryptophan fluorescence. For tryptophan fluorescence analysis, 10 µM wild-type or mutant AvrA protein was prepared in buffer containing 20 mM Tris, pH 7.5, 150 mM NaCl and 5% glycerol. The proteins were excited at 280 nm, and the emission at 330 nm was monitored at 25 °C with a Tecan Infinite 200 PRO plate reader. IP6 was added at a protein/IP6 ratio of 1:2, and decreases in fluorescence were monitored over time. The fluorescence in relative fluorescence units (RUU) was calculated on the basis of the fluorescence at the indicated time point relative to the fluorescence at the beginning of the experiment.

1D 1H NMR spectroscopy. NMR experiments were carried out as previously described. Briefly, 0.1 mM purified wild-type or mutant HopZ1a protein in the absence or presence of IP6 (protein/IP6 ratio of 1:10) was dissolved in 500 µL buffer containing 20 mM sodium phosphate, pH 7.5, 150 mM NaCl and 10% D2O. NMR spectra were collected with a Bruker Advance 700-MHz NMR spectrometer equipped with a cryogenic TXI probe at 25 °C.

Homology modeling of AvrA. Homology models of AvrA were obtained with Modeller 9v15 by using the crystal structure of the Hop21a–IP3 complex. The initial 3D sequence alignments between AvrA and the template structures were performed with Strap34. Highly conserved residues were manually adjusted for proper alignment. A total of 10,000 models were generated with a fully annealed protocol, and the optimal model was chosen according to the Discrete Optimized Protein Energy (DOPE) score.

Molecular dynamics (MD) simulation. HopZ1a was prepared with the Protein Preparation Tool (ProPrep) in the Schrödinger 2015 suite. Asparagine, glutamine and histidine residues were automatically checked for flips. Hydrogen atoms were added to the HopZ1a model according to the physiological pH environment with PROPKA35 in Maestro (Schrödinger) along with an optimized hydrogen-bond network. MD simulations were performed in Desmond (https://www.deskawaresearch.com/resources/desmond.html), Proteins, ions and water molecules were parameterized with the CHARMM22star force-field parameter set, whereas the CHARMM CGenFF small-molecule force field was used for the ligands, which were analyzed in GAUSSIAN 09 (Gaussian) for structure optimization at the B3LYP/6-31G* level before generation of force-field parameters. Bond lengths to hydrogen atoms in each protein system were constrained with M-SHAKE38. Van der Waals and short-range electrostatic interactions were cut off at 10 Å. 400-ns (2 × 200) MD simulations were performed for both HopZ1a and ligand-bound HopZ1a. Results were analyzed with VMD39, Gromacs40 and bio3D41.

Correlation network analysis. Correlated atomic fluctuations of a particular protein state were characterized, as reported elsewhere, with Bio3D. The network nodes represent residues, which are connected through edges weighted according to their constituent atomic correlation values. Community analysis and node centrality with Bio3D and suboptimal path calculation with WISP44 were performed on each network to characterize network properties and to identify residues involved in the dynamic coupling of distal sites. The parameters for the suboptimal path analysis included input source and sink nodes, as well as the total number of paths to be calculated. The latter parameter was set to 500 paths, which yielded convergent results in all cases.

Normal mode analysis (NMA). To predict the functional motions of HopZ1a, the NMA calculations were performed with the ProDy package45, with the MD simulation trajectories of HopZ1a and HopZ1a–IP6 as input.