A 1-phytase type III effector interferes with plant hormone signaling

Doreen Blüher, Debabrata Laha, Sabine Thieme, Alexandre Hofer, Lennart Eschen-Lippold, Antonia Masch, Gerd Balcke, Igor Pavlovic, Oliver Nagel, Antje Schonsky, Rahel Hinkelmann, Jakob Wörner, Nargis Parvin, Ralf Greiner, Stefan Weber, Alain Tissier, Mike Schutkowski, Justin Lee, Henning Jessen, Gabriel Schaaf, & Ulla Bonas

Most Gram-negative phytopathogenic bacteria inject type III effector (T3E) proteins into plant cells to manipulate signaling pathways to the pathogen’s benefit. In resistant plants, specialized immune receptors recognize single T3Es or their biochemical activities, thus halting pathogen ingress. However, molecular function and mode of recognition for most T3Es remains elusive. Here, we show that the Xanthomonas T3E XopH possesses phytase activity, i.e., dephosphorylates phytate (myo-inositol-hexakisphosphate, InsP$_6$), the major phosphate storage compound in plants, which is also involved in pathogen defense. A combination of biochemical approaches, including a new NMR-based method to discriminate inositol polyphosphate enantiomers, identifies XopH as a naturally occurring 1-phytase that dephosphorylates InsP$_6$ at C1. Infection of Nicotiana benthamiana and pepper by Xanthomonas results in a XopH-dependent conversion of InsP$_6$ to InsP$_5$. 1-phytase activity is required for XopH-mediated immunity of plants carrying the Bs7 resistance gene, and for induction of jasmonate- and ethylene-responsive genes in N. benthamiana.

 DOI: 10.1038/s41467-017-02195-8

1 Institute for Biology, Department of Genetics, Martin-Luther University Halle-Wittenberg, Weinbergweg 10, 06120 Halle (Saale), Germany. 2 Institute of Crop Science and Resource Conservation, Department of Plant Nutrition, University of Bonn, Karlrobert-Kreiten-Strasse 13, 53115 Bonn, Germany. 3 Center for Plant Molecular Biology, Department of Plant Physiology, Eberhard Karls University Tübingen, Auf der Morgenstelle 32, 72076 Tübingen, Germany. 4 Department of Chemistry, University of Zurich, Winterthurerstrasse 190, 8057 Zurich, Switzerland. 5 Department of Stress and Developmental Biology, Leibniz Institute of Plant Biochemistry, Weinberg 3, 06120 Halle (Saale), Germany. 6 Institute for Biochemistry and Biotechnology, Department of Enzymology, Martin-Luther University Halle-Wittenberg, Kurt-Mothes-Strasse 3, 06120 Halle (Saale), Germany. 7 Department of Cell and Metabolic Biology, Leibniz Institute of Plant Biochemistry, Weinberg 3, 06120 Halle (Saale), Germany. 8 Institute of Organic Chemistry, Albert-Ludwigs University Freiburg, Albertstrasse 21, 79104 Freiburg, Germany. 9 Institute of Physical Chemistry, Albert-Ludwigs University Freiburg, Albertstrasse 21, 79104 Freiburg, Germany. 10 Department of Food Technology and Bioprocess Engineering, Max-Rubner-Institut, Federal Research Institute of Nutrition and Food, Haid-und-Neu-Straße 9, 76131 Karlsruhe, Germany. Doreen Blüher and Debabrata Laha contributed equally to this work. Correspondence and requests for materials should be addressed to H.J. (email: henning.jessen@oc.uni-freiburg.de) or to G.S. (email: gabriel.schaaf@uni-bonn.de) or to U.B. (email: ulla.bonas@genetik.uni-halle.de)
Gram-negative phytopathogenic xanthomonads infect a broad range of plant species causing substantial crop yield losses. Pathogenicity depends in most cases on a conserved type III secretion (T3S) system that translocates effector proteins directly into the plant cell cytosol\(^1\). The pepper and tomato pathogen *Xanthomonas campestris* pv. *vesicatoria* (Xcv, also termed *X. euvesicatoria*)\(^2\) encodes more than 30 type III effector (T3E) proteins, designated Xops (*Xanthomonas* outer proteins), whose collective action in host cells results in bacterial spot disease\(^3,4\). In resistant plant cultivars, single effectors are recognized by specific immune receptors\(^1\) often inducing the hypersensitive response (HR), a rapid, local programmed cell death at the infection site which restricts pathogen ingress\(^5\). While the molecular functions of most T3Es from *Xanthomonas* are elusive, members of the large family of transcription activator-like (TAL) effectors act as transcription factors in the plant cell\(^6\). Other T3Es display enzymatic activities such as the E3 ubiquitin ligase XopL7 or AvrBsT, a member of the YopJ/AvrRxv family of effector (T3E) proteins, designated Xops (plant-encoded type III secretion (T3S) system that translocates effector proteins directly into the plant cell\(^1\)). The pepper and tomato pathogen *X. euvesicatoria* is the petiole blight pathogen of *Capsicum* (peppers, tomatoes, and related species), causing a disease known as spot disease\(^3,4\). In resistant plant cultivars, single effectors are recognized by specific immune receptors\(^1\) often inducing the hypersensitive response (HR), a rapid, local programmed cell death at the infection site which restricts pathogen ingress\(^5\).

**Results**

**XopH has weak protein phosphatase activity.** Using optimized buffer conditions (Supplementary Fig. 1a), we determined the essential amino acid residues for XopH-mediated phosphatase activity on pNPP: H239 in the WPD loop and C267 in the active site (Supplementary Fig. 1b). Similar results were obtained with the phosphotyrosine-containing peptide pTyr2, the best substrate out of six tested commercial phosphopeptides (Supplementary Fig. 1c, d). The XopH N-terminal region harbors two proline-rich regions (PRRs), putative peptide/protein interaction sites\(^11\) that might be involved in substrate recognition (Fig. 1a). Mutations in both PRR motifs compromised catalytic activity albeit less in case of the PRR1 motif. Deletion of the first 77 amino acid residues led to a complete loss of protein phosphatase activity (Supplementary Fig. 1d). To determine XopH substrate specificity, high-density peptide microarrays comprising more than 6000 pTyr peptides were incubated with XopH and the catalytically inactive C267A variant, respectively (for details see “Methods” section). The top 72 XopH substrates showed >70% cleavage by WT XopH and were compared to the negative sample set represented by all peptides displayed on the array. The resulting two-sample logo is shown in Supplementary Fig. 1e. Next, kinetic constants of XopH protein phosphatase activity were determined using three

![Image](158x291 to 343x415)

**Fig. 1** XopH possesses phytate-degrading activity that is required for HR induction. **a** Amino acid (aa) sequence alignment of XopH with the Selenomomas *ruminantium* phytase generated with T-Coffee\(^66\). Identical and similar aa are shaded black and gray, respectively, using Boxshade\(^67\). Dashes indicate gaps. Catalytic residues in the WPD and P loops are marked by asterisks. Proline-rich regions (PRRs), putative peptide/protein interaction sites\(^11\) are boxed. **b** XopH protein structure modeled after the *S. ruminantium* phytase crystal structure (pdb 1U24) using Phyre2\(^28\), visualized by PyMol\(^68\). Blue, phytase domain; gray, N-terminal domain (aa 1–77). **c** InsP\(_6\) dephosphorylation by XopH (WT) and mutants, respectively. PRR1, P48,52,53A; PRR2–1, P69,71A; PRR2–2, P73,74,75,76A; CH, H239A/C267A; Del2–77, deletion of aa 2–77. GFP served as negative control. Values are means of two technical replicates. Error bars indicate s.d. The experiment was performed twice with similar results, using two independent protein preparations each. **d** HR induction in pepper ECW-70R (*Bs7*) leaves after *Agrobacterium*-mediated expression of XopH and mutant variants. Two days post inoculation (dpi), leaves were bleached in ethanol for better visualization of the HR. **e** Protein expression, two dpi, in the same plants analyzed in **d**. Immunoblot signals at expected sizes are marked by asterisks. The experiment was repeated twice with similar results.
two independent protein preparations each
MgCl₂ (open circles) are 40.0 raising the possibility that protein
myo Phytases dephosphorylate in absence and presence of 1 mM metal ions or 1 mM EDTA. Reaction buffer contained 10 mM NaCl.

dephosphorylation does not represent XopH Phyre214 revealed a high similarity to the phytase PhyA from the
anaerobic bacterium Selenomonas ruminantium15 (Fig. 1b). Phytases dephosphorylate myo-inositol hexakisphosphate (InsP₆, synonym.: phytate) to lower phosphorylated myo-inositol derivatives and ortho-phosphate. Phytate is the major phosphate storage compound in plant seeds16 and is involved in plant defenses against viral, bacterial, and fungal pathogens17,18. We tested InsP₆ dephosphorylation by XopH in vitro at pH 7.0, which resembles physiological conditions for XopH activity because the protein is localized in both the nucleus and cytoplasm of the plant cell19. As shown in Fig. 1c, XopH indeed dephosphorylated InsP₆ 10 mM NaCl 110.6 138.3 40.0 ± 5.1 138.3 ± 15.0 72.9 ± 13.7 45.3 2.66 × 10⁵ 0.99

---

**Table 1 Catalytic constants of tyrosine phosphatase and phytase activity of XopH**

| Substrate | Additive | \( V_{\text{max}} \) (µmol L⁻¹ min⁻¹) | \( K_M \) (µM) | \( k_{\text{cat}} \) (s⁻¹) | \( k_{\text{cat}}/K_M \) (M⁻¹ s⁻¹) | \( R^2 \) |
|-----------|----------|-------------------------------------|----------------|----------------|-------------------------------|------|
| pTyr2     | 10 mM NaCl, 1 mM MgCl₂ | 0.63 ± 0.16 | 25.7 ± 12.2 | 0.009 | 3.50 × 10² | 0.89 |
| pTyr-chip | 10 mM NaCl, 1 mM MgCl₂ | 0.21 ± 0.12 | 29.7 ± 3.0 | 0.028 | 9.42 × 10² | 0.99 |
| InsP₆     | 100 mM NaCl, 1 mM MgCl₂ | 40.0 ± 5.1 | 72.9 ± 13.7 | 19.4 | 2.66 × 10⁵ | 0.99 |
| InsP₆     | 10 mM NaCl | 110.6 ± 9.5 | 138.3 ± 15.0 | 45.3 | 3.28 × 10⁵ | 0.99 |

*Activity was measured in 50 mM HEPES (pH 7.0) under reducing conditions as described in “Methods” section.

---

Fig. 2 Characterization of the XopH phytase activity. a XopH activity in presence of 10 mM NaCl in different buffering systems. b XopH activity in presence of 100 mM NaCl and 1 mM MgCl₂ at different temperatures (16, 25, 37, 50, and 60 °C). c XopH activity in buffer with 10–1000 mM NaCl. d XopH activity in absence and presence of 1 mM metal ions or 1 mM EDTA. Reaction buffer contained 10 mM NaCl. e XopH-dependent dephosphorylation of 0.1 mM InsP₆, glucose-6-phosphate (G6P), glycerol phosphate (GlyP), and fructose 1,6-bisphosphate (F1,6BP), respectively. f Kinetics of InsP₆ dephosphorylation by XopH. Data were fitted to Michaelis Menten equation using KaleidaGraph4.0 (www.synergy.com). \( V_{\text{max}} \) and \( K_M \) in presence of 100 mM NaCl and 1 mM MgCl₂ (open circles) are 40.0 ± 5.1 pmol µg⁻¹ min⁻¹ and 72.9 ± 13.7 µM, and in presence of 10 mM NaCl (closed circles) 110.6 ± 9.5 pmol µg⁻¹ min⁻¹ and 138.3 ± 15.0 µM. Values are means of two technical replicates. Error bars indicate s.d. The experiments were performed twice with similar results, using two independent protein preparations each.

**XopH degrades phytate.** XopH structure predictions using Phyre214 revealed a high similarity to the phytase PhyA from the anaerobic bacterium Selenomonas ruminantium15 (Fig. 1b). Phytases dephosphorylate myo-inositol hexakisphosphate (InsP₆, synonym.: phytate) to lower phosphorylated myo-inositol derivatives and ortho-phosphate. Phytate is the major phosphate storage compound in plant seeds16 and is involved in plant defenses against viral, bacterial, and fungal pathogens17,18. We tested InsP₆ dephosphorylation by XopH in vitro at pH 7.0, which resembles physiological conditions for XopH activity because the protein is localized in both the nucleus and cytoplasm of the plant cell19. As shown in Fig. 1c, XopH indeed dephosphorylated phytate. To the best of our knowledge, XopH is the first type III effector with phytate-degrading activity to be reported. The WPD loop mutant H239A retained residual catalytic activity on InsP₆, whereas the P loop mutant C267A and the double mutant H239A, C267A (CH) displayed a complete loss of function (Fig. 1c). Since mutations in the PRR motifs and deletion of the first 77 amino acids of XopH had only minor effects on its lytic activity on InsP₆, whereas the P loop mutant C267A and the double mutant H239A, C267A (CH) displayed a complete loss of function (Fig. 1c). Since mutations in the PRR motifs and deletion of the first 77 amino acids of XopH had only minor effects on its lytic activity on InsP₆, whereas the P loop mutant C267A and the double mutant H239A, C267A (CH) displayed a complete loss of function (Fig. 1c). Since mutations in the PRR motifs and deletion of the first 77 amino acids of XopH had only minor effects on its
T3Es, which contain a structurally disordered N-terminal region carrying signals for type III-dependent secretion and translocation, and at least one domain for subcellular localization and function in the host cell. Detailed characterization of the XopH phytate-degrading activity revealed a broad pH activity profile with an optimal pH range of 5–7 depending on the buffer used, as well as a rather low temperature optimum of 25 °C compared to other phytases. In addition, we tested the effects of ionic strength and different metal ions. XopH activity is highest at low salt condition and inhibited by Zn\(^{2+}\), Mn\(^{2+}\), and partially by Ca\(^{2+}\) and Mg\(^{2+}\), but not by the metal ion chelator EDTA. There was no detectable XopH-dependent dephosphorylation of the alternative phytase substrates glucose-6-phosphate, glycerol phosphate, and fructose 1,6-bisphosphate suggesting a high substrate specificity of XopH.

Using optimized conditions, the specificity constant \(k_{cat}/K_m\) was found to be 3.28 × 10\(^5\) M\(^{-1}\) s\(^{-1}\) (Fig. 2f and Table 1), thus three orders of magnitude higher than the dephosphorylation \(k_{cat}/K_m\) of the best peptide substrate "pTyr-chip" and comparable to the activity of the S. ruminantium phytase. We conclude, therefore, that the primary enzymatic activity of XopH is that of a phytase. Notably, mutant analyses showed that the XopH-induced HR in pepper plants containing the Bs7 resistance (R) gene depends on the phytase but not protein phosphatase activity.

Fig. 3 Both cytoplasmic and nuclear-localized XopH variants are biologically active. a Confocal laser scanning microscopy of N. benthamiana leaves two dpi with Agrobacterium-mediating expression of GFP, XopH, or XopH fused to a nuclear localization signal (NLS), a nuclear export signal (NES), and a mutated NES (nes), respectively. DAPI (4',6'-Diamidino-2-phenylindole) staining indicates nuclei. b HR induction by XopH and derivatives. The same Agrobacterium strains analyzed in a were infiltrated into leaves of resistant pepper ECW-70R plants. Leaves were harvested two dpi and bleached with ethanol. c Agrobacterium-mediated expression of His\(_6\)-tagged GFP fusion proteins. Samples were harvested two dpi from the same areas as investigated in a and analyzed by immunoblot. Signals at expected sizes are marked by an arrow. d SAX-HPLC profiles of extracts from \(^{[3H]}\)-myo-inositol-labeled transgenic N. benthamiana seedlings that were inoculated with the Agrobacterium strains described in a. The experiments were repeated twice (a-c) and once (d), respectively, with similar results.
suggested that Bs7 recognizes the consequence of XopH activity, e.g., changes in myo-inositol polyphosphates, rather than the protein itself. This hypothesis is further corroborated by the fact that changes in the intracellular localization of XopH by fusion to nuclear localization or export signals (NLS, NES) do not significantly affect the HR induction (Fig. 3a–c).

**XopH is a 1-phytase.** One possibility to classify phytases is based on the number assigned to the carbon atom of the myo-inositol ring at which the first dephosphorylation takes place. Myo-inositol itself is a meso-compound with a plane of symmetry dissecting the 2 and 5 positions (Fig. 4a). This plane of symmetry is retained in InsP₆, however, dephosphorylation at either the 1/3 or 4/6 position will create mirror images (enantiomers) by breaking local symmetry (Supplementary Fig. 2). While plants often express 4-phytases that first target the phosphate at C position 4, activities of all phytase types have been detected in nature, with the exception of 1-phytases. Polycrylamide gel electrophoresis (PAGE) revealed that the XopH reaction product had the same electrophoretic mobility as the enantiomeric 1-OH and 3-OH InsP₅ isomers (Fig. 4a). InsP₅ [1/3-OH] isomer identity was corroborated by spiking experiments (Supplementary Fig. 4a) and ion chromatography (Fig. 4c and Supplementary Fig. 3e). To elucidate enantiomer identity of the XopH reaction product, we investigated susceptibility of all InsP₅ isomers to degradation by XopH. Except for InsP₅ [1-OH], all isomers (in particular InsP₅ [3-OH]) were readily degraded, albeit the 2-OH and the 6-OH isomer with lower efficiency (Supplementary Fig. 4b). Importantly, resistance of commercial InsP₅ [1-OH] to XopH-mediated dephosphorylation was not caused by a contaminating phytase inhibitor since addition of InsP₅ to the InsP₅ [1-OH]/XopH reaction mixture resulted in rapid hydrolysis of InsP₅ to InsP₅ (Supplementary Fig. 4c). These results suggest that InsP₅ [1-OH] represents the product of XopH-mediated InsP₅ hydrolysis.

**A new NMR-based method confirms InsP₅ [1-OH] as XopH product.** To unambiguously reveal isomer identity of the XopH cleavage product, we developed a novel method based on nuclear magnetic resonance (NMR) spectroscopy, which can be applied to samples in the presence of buffers and salts that do not interfere with ³¹P-NMR. As briefly discussed above, myo-inositol and phytate are meso-compounds displaying an internal plane of symmetry (Fig. 4a and Supplementary Fig. 2). Thus, for phytate a ³¹P-NMR shows two distinct resonances for the phosphates in the 2 and 5 positions, one for the two phosphates in the 1 and 3 positions and another one for the 4 and 6 positions due to internal symmetry. The integrals of the resonances, therefore, display a ratio of 1 (position 2):2 (positions 1 and 3):2 (positions 4 and 6):1 (position 5) (Supplementary Fig. 5a, b). Breaking the symmetry by dephosphorylation in either the 1/3 or 4/6 position will result in distinct signals for every single phosphate, even though they may accidentally have the same chemical shift. Using NMR, one cannot discriminate enantiomers, as they will generate identical resonances in an environment that is achiral (Fig. 5a, top trace). Our method is based on the addition of an enantiopure counter ion that forms one or multiple ion pairs with the inositol phosphate giving rise to diastereomeric salts. This will generate different local environments for the atoms and, therefore, different chemical shifts of the resonances. Such strategies have been developed for other acidic compounds mainly based on ¹H-NMR. Since phosphates associate tightly with guanidinium groups, we screened several candidate compounds and identified L-arginine amide hydrochloride salt (L-Arg-N) as a suitable counter ion to generate diastereomeric ion pairs with InsP₅ even in aqueous buffer. Separate ³¹P-NMR measurements of both InsP₅ [1-OH] and InsP₅ [3-OH] in the presence of excess L-Arg-N resulted in slightly different shifts for all five peaks (Supplementary Fig. 6). Since ³¹P-NMR chemical shifts are very sensitive to the matrix and especially small changes in pH, we performed spiking experiments mixing InsP₅ [1-OH] and InsP₅ [3-OH] in different ratios (1:1.5, 1:1, and 1.5:1). While the chiral non-racemic mixture showed four peaks in a ratio of 1:1:2:1, addition of an excess of L-Arg-N caused a large shift in all resonances and led to resolvable differences for four of the five phosphate signals of both enantiomers (Fig. 5a). This effect was observed in all three different ratios measured. Thus, ³¹P-NMR can be used to identify enantiomeric inositol phosphates in the presence of excess L-Arg-N as chiral solvating agent.

We used our method to analyze the XopH hydrolysis product without purification in phosphate-free buffer. Furthermore, commercial InsP₅ [1-OH] was added to the reaction mixture. The spectrum showed the expected five resonances for InsP₅ and six possible InsP₅ isomers revealed that the XopH cleavage product displays the same electrophoretic mobility as the enantiomeric 1-OH and 3-OH InsP₅ isomers (Fig. 4a). InsP₅ [1/3-OH] isomer identity was corroborated by spiking experiments (Supplementary Fig. 4a) and ion chromatography (Fig. 4c and Supplementary Fig. 3e). To elucidate enantiomer identity of the XopH reaction product, we investigated susceptibility of all InsP₅ isomers to degradation by XopH. Except for InsP₅ [1-OH], all isomers (in particular InsP₅ [3-OH]) were readily degraded, albeit the 2-OH and the 6-OH isomer with lower efficiency (Supplementary Fig. 4b). Importantly, resistance of commercial InsP₅ [1-OH] to XopH-mediated dephosphorylation was not caused by a contaminating phytase inhibitor since addition of InsP₅ to the InsP₅ [1-OH]/XopH reaction mixture resulted in rapid hydrolysis of InsP₅ to InsP₅ (Supplementary Fig. 4c). These results suggest that InsP₅ [1-OH] represents the product of XopH-mediated InsP₅ hydrolysis.
an additional resonance for phosphate that was easily identified by (i) recording of a proton-coupled spectrum (Fig. 5b, lower trace) and (ii) integration of the peaks (Fig. 5c). The proton-coupled spectrum showed peak splitting (doublets) only for the phosphates bound to the inositol scaffold, whereas in PO₄³⁻ no bound protons were available for coupling. The spiked spectrum also showed that the integral of PO₄³⁻ was smaller than those of the other resonances, as only InsP₅ [1-OH] was added. Heating of the sample to destroy residual XopH activity did not cause decomposition of InsP₅ (Fig. 5c). Next, a XopH digest obtained as described above was inactivated at 95 °C and then spiked with InsP₅ [3-OH] in different ratios in presence of an excess of L-Arg-N in excess (ca. 100-fold). The mixture was heated to 95 °C for 15 min to denature residual XopH. Integration identifies the phosphate resonance (expected ratio is 1:0.6) and shows that no decomposition after boiling takes place.

**Fig. 5** ³¹P-NMR in the presence of L-arginine amide identifies InsP₅ [1-OH] as the XopH reaction product. **a** Upper trace: Mixture of InsP₅ [1-OH] (A) and InsP₅ [3-OH] (B) in different ratios in ammonium acetate buffer (pH 7.1) in the absence of L-arginine amide (L-Arg-N). No peak separation was observed and integration gives the expected values 1:2:1 (from left to right). Addition of L-Arg-N in excess (ca. 100-fold) leads to separation of the resonances of InsP₅ [1-OH] (A) or InsP₅ [3-OH] (B) in all three different ratios studied (1:1.5, 1:1, and 1.5:1). Asterisks mark an impurity. **b** Digest of 600 nmol InsP₆ by XopH in ammonium acetate buffer (pH 7.1), spiking with 400 nmol InsP₅ [1-OH] and addition of L-Arg-N in excess (ca. 100-fold). No additional peaks can be seen in a proton-decoupled spectrum (upper trace). A proton-coupled spectrum identifies all resonances belonging to inositol-bound phosphates (A). **c** Digest of 600 nmol InsP₆ by XopH, spiking with InsP₅ [1-OH] and addition of L-Arg-N in excess (ca. 100-fold). The mixture was heated to 95 °C for 15 min to denature residual XopH. Integration identifies the phosphate resonance (expected ratio is 1:0:0.6) and shows that no decomposition after boiling takes place. **d** XopH digest of 600 nmol and excess L-Arg-N (ca. 100-fold) boiled at 95 °C for 15 min. Subsequent addition of InsP₅ [3-OH] (B) in different ratios leads to appearance of new resonances.

XopH leads to InsP₅ accumulation in vivo. To analyze the consequences of XopH phytate-degrading activity in vivo, we compared the composition of myo-inositol polyphosphates in *Saccharomyces cerevisiae* ectopically expressing XopH or the phytase-inactive XopHC267A mutant, by strong anion exchange (SAX) HPLC, a method that by itself does not allow to discriminate between enantiomers in the absence of chiral selectors. Ectopic expression of XopH in yeast caused a strong reduction of InsP₆ and a robust accumulation of InsP₅ with the chromatographic mobility of InsP₅ [1/3-OH] (Fig. 6). Furthermore, an increase in one InsP₄ and one InsP₃ species, both of unknown stereochemistry, was detected. By contrast, expression of the catalytically dead mutant XopHC₂₆₇ₐ did not cause significant changes in the HPLC profile as compared to the control (Fig. 6).

In agreement with a role of XopH in InsP₆ dephosphorylation, transgenic *Nicotiana benthamiana* plants constitutively expressing *xopH* showed a robust accumulation of InsP₅ [1/3-OH] at the cost of InsP₆ (Fig. 7a and Supplementary Fig. 7). Altered localization of XopH to only nucleus or cytoplasm had no major effect on its ability to hydrolyze InsP₆ (Fig. 3d), a small molecule likely to diffuse freely between nucleo- and cytoplasm. Given the biochemical data described above, the observed InsP₅c isomer likely represents InsP₅ [1-OH]. To test this idea, InsP₅c and InsP₆ were purified from [³¹P]-myo-inositol-labeled *xopH*- and GFP-transgenic *N. benthamiana* seedlings, subjected to digest by XopH.
Deletion of xopQ turns Xcv strain 85-10 into a N. benthamiana pathogen that grows in the tissue and elicits typical disease symptoms, i.e., water-soaked lesions. As shown in Fig. 7c, inoculation of 85-10ΔxopQ ("WT") induced a slight decrease in the plant's InsP₆ content and an additional InsP₅ signal. By contrast, leaves inoculated with the respective xopH frameshift mutant (85-10ΔxopQ_fs-xopH) contained even more InsP₆ than mock-infiltrated plant tissue and no InsP₅ in detectable amounts. 85-10ΔxopQ_fs-xopH was complemented by re-integration of xopH into the genome (Fig. 7c).

In addition, we analyzed XopH-dependent changes in InsP₆ and InsP₅ contents in the Xcv-pepper system. Although InsP₆ was barely detectable in pepper, there was a clear InsP₆ decrease after inoculation of strains expressing XopH, but not the inactive XopH_C267A mutant version (Supplementary Fig. 7b). Notably, the xopH frameshift mutant was complemented by expression of XopH_DeltΔT₇ confirming that the phytase, and not the protein phosphatase, activity is responsible for the observed InsP₆ decrease. Leaf tissue inoculated with a T3S-deficient strain (ΔhrcN) showed a similar InsP₆ content as that infected with the xopH mutant. This suggests that XopH is the only T3E in Xcv strain 85-10, which significantly affects phytate levels.

XopH affects plant hormone pathways. Plant hormone signaling pathways can involve InsP₆ co-factors, as was suggested for auxin and jasmonate (JA) signal transduction, and might, therefore, be affected by changes in the InsP₆ homeostasis. XopH expression in N. benthamiana leaves causes a strong concomitant reduction of inositol pyrophosphates InsP₇ and InsP₈ (Fig. 7a and Supplementary Fig. 7). The latter is required for jasmonate perception, most likely by inducing an allosteric switch of the jasmonate receptor complex. We analyzed the influence of XopH expression on transcript abundance of JA-responsive genes in N. benthamiana leaves without or 20 min after wounding. qRT-PCR analysis revealed that XopH led to a slightly reduced expression of JA-responsive genes in N. benthamiana leaves with or without wounding, which were used as general JA marker genes in previous studies in solanaceous plants. Weaker induction was observed with XopHDel77, which is consistent with the reduced activity of this XopH variant (Fig. 1c, d) and demonstrates that the phytase, and not the protein phosphatase, activity is required for gene induction (Fig. 7a, b).

Notably, PR1b, PR4, and PI-II genes are not only JA responsive, but also ethylene (ET) responsive, suggesting that XopH might affect the ET pathway. Consistent with this idea, transgenic N. benthamiana plants constitutively expressing xopH were significantly smaller than transgenic GFP control plants of the same age and showed signs of early senescence (Supplementary Fig. 8). We performed virus-induced gene silencing of PR1b, PR4, and PI-II (Fig. 8a, b), which were used as general JA marker genes in previous studies in solanaceous plants. Deletion of xopQ turned Xcv strain 85-10 into a N. benthamiana pathogen, which triggers a defense reaction and prevents bacterial growth. XopH is a T3E with a novel phytate-degrading activity, which we demonstrated in vitro and in planta. Using a combination of different digestion experiments of chemically pure inositol polyphosphates and an NMR-based method to discriminate InsP₅ and InsP₆, and separated by SAX-HPLC. Under these conditions, XopH did not further dephosphorylate InsP₅c, whereas purified InsP₆c was readily degraded, thus increasing the InsP₆c peak (Fig. 7b and Supplementary Fig. 8). This is consistent with the XopH-dependent InsP₅c corresponding to InsP₅ [1-OH], whereas InsP₅b represents InsP₅ [4/6-OH], and InsP₅a represents InsP₅ [1/3-OH]. The isomeric natures of InsP₅c, InsP₅b, InsP₅a, and InsP₅ are unknown. 

Fig. 6 XopH changes InsP₆ contents in yeast. a HPLC profile of extracts from yeast transformants labeled with [³H]-myo-inositol. ev empty vector. Based on published chromatographic mobilities, InsP₅a represents InsP₅ [2-OH], InsP₅b represents InsP₅ [4/6-OH], and InsP₅a represents InsP₅ [1/3-OH]. The isomeric natures of InsP₅c, InsP₅b, InsP₅a, and InsP₅ are unknown. b Zoom-in on the HPLC profile. c Relative amounts of InsP₆ and InsP₅ in the yeast transformants. Error bars indicate s.e.m. The experiment was repeated independently with similar results.
isomers, we identified XopH as the prototype of a new class of phytases initiating \( \text{InsP}_6 \) dephosphorylation at C position 1. Thus, XopH belongs to the relatively small number of \textit{Xanthomonas} T3Es with identified biochemical activities: the large group of TAL effectors and T3Es with uridylyltransferase (AvrAC), acetyltransferase/cysteine protease (YopJ/AvrRxv family), SUMO protease (XopD), and ubiquitin ligase activity (XopL)\(^1\).

Our mutant analyses indicate that the phytase and not the protein phosphatase activity is relevant for XopH’s biological activities, i.e., HR elicitation in the host plant pepper (Fig. 1d) and gene induction in \textit{N. benthamiana} (Fig. 8a). In this respect, XopH has a different physiological function than its homolog HopAO1 (formerly termed HopPtoD2; 25% identical to XopH) from \textit{Pseudomonas syringae pv. tomato}. HopAO1 was reported to...
reduce tyrosine phosphorylation of the PAMP receptor EFR, after recognition of the elf18 peptide from bacterial elongation factor Tu, thus preventing immune responses. By contrast, XopH does not inhibit elf18-inducible gene expression. Furthermore, membrane proteins, such as EFR (or other proteins that do not enter the nucleus), are unlikely targets of XopH, because nuclear or cytoplasmic XopH variants exhibit similar HR-inducing activities in resistant pepper plants (Fig. 3), and both virulence and HR-inducing activities of T3Es are often connected (see below). Notably, we found that HopAO1 degraded phytate in vitro but, in contrast to XopH, to lower phosphorylated myo-inositol derivatives (Supplementary Fig. 11). In conclusion, this suggests different activities for XopH and HopAO1.

The fact that only the phytochrome domain of XopH is essential for HR induction suggests that Bs7 recognizes the product of the enzyme reaction, i.e., InsP₅ [1-OH], or changes caused by altered inositol polyphosphate homeostasis rather than the XopH protein itself. This hypothesis is supported by the fact that XopH variants solely localizing to the nucleus or the cytoplasm of the plant cell have the same HR-inducing activity as the WT protein, which correlates with similar phytoase activities (Fig. 3). Indeed, most R proteins recognize their cognate T3Es indirectly by detecting effector-triggered changes in plant targets (guard model) or non-functional target mimics (decoy model). Notably, the molecules targeted by T3Es are usually proteins or, as in case of TAL effectors, DNA sequences and not low-molecular substances like inositol polyphosphates.

What are the consequences of phytate dephosphorylation and InsP₅ [1-OH] accumulation for susceptible plant cells and the bacterial pathogen, which lives in the intercellular spaces? One possibility is that XopH liberates phosphate from the plant tissue to improve the nutritional status of the pathogen. A role in phosphate acquisition from the extracellular milieu has been reported for the putative phytase PhyA secreted by the rice pathogen X. oryzae pv. oryzae. PhyAox enhances bacterial growth in medium with phytate as sole phosphate source and is required for virulence. Another scenario supported by our qRT-PCR data is that XopH interferes with plant hormone pathways, probably by affecting the abundance of potential co-factors. According to this hypothesis, our data give a first hint that InsP₅ co-factors are also involved in the ET pathway. Since ET acts synergistically with JA to regulate resistance against necrotrophic pathogens, stimulation of the ET pathway might be beneficial to hemibiotrophic bacteria like Xcv. Furthermore, bacterial dispersal may benefit from ET functions in disease development at later infection stages. Notably, XopH has been recently reported to promote disease symptom formation on tomato. A third model is that XopH compromises plant defense mechanisms by degradation of InsP₅, which has been suggested to play a role in resistance against a wide variety of pathogens. XopH might sequester InsP₅ by degrading it to an InsP₃ isomer, which is not easily metabolized by the plant (as shown above) and accumulates. Indeed, XopH has been recently shown to inhibit defense reactions, e.g., callose deposition, in Arabidopsis. A similar strategy is imaginable for the phytase LppA from the human pathogen Legionella pneumophila: while the product of LppA activity is unknown, LppA has been shown to be translocated via the type IV secretion system and to counteract intracellular bacterial growth restriction by phytate. By taking advantage of an enantiomer-specific Dictyostelium InsP₆ [3-OH] kinase activity, the InsP₅c species in mung bean seedlings was proposed to largely consist of InsP₅ [1-OH]. Future work will have to clarify isomer identity and physiological role of the endogenous (XopH-independent) InsP₅c species in N. benthamiana and pepper. Because InsP₅ is ubiquitous in eukaryotes and is known to have critical roles in these systems, we believe that the mechanism employed by Xanthomonas could be a common strategy of host immune system avoidance for bacteria and other pathogens.

In addition, the novel activity of XopH reported here adds to the molecular toolbox for future biotechnological manipulation of phytate levels. Because of its high affinity for minerals including iron and zinc ions, phytate is considered an antimutagen in humans and monogastric animals (swine, poultry, and fish), all of which lack the ability to significantly degrade phytate. Hence, phytases are economically important enzymes that are widely used to improve phosphate and mineral availability in animal feed and to reduce phosphate excretion by animals. Furthermore, the novel 1-phytase specificity of XopH gives access to a new set of inositol phosphate isomers. InsP₅ [1-OH] and its derivatives generated by other phytases will, for instance, prove useful for the future characterization of new phytases and phytosphosphates.

**Methods**

**Plant material and inoculations.** Nicotiana benthamiana and pepper (Capsicum annuum) cultivar ECW-70R (Bt70) plants were grown in the greenhouse under standard conditions (day and night temperatures of 25 °C and 19 °C, respectively, for N. benthamiana, and 25 °C and 19 °C for pepper, with 16 h light and 40–60% humidity). For in planta protein expression, mature leaves of 5- to 7-week-old plants were inoculated with Agrobacterium tumefaciens at OD₆₀₀ 0.8 in infiltration medium (10 mM MES pH 5.5, 10 mM MgCl₂, 150 μM acetylsyringone) using a needleless syringe. Xcv was inoculated (in 10 mM MgCl₂; OD₆₀₀ = 0.1 or 0.2) into leaves of 6-week-old pepper or N. benthamiana plants using a needleless syringe. Inoculated pepper plants were transferred to a Percival growth chamber (Percival Scientific, Perry, USA).

**Bacterial strains and growth conditions.** Escherichia coli BL21(DE3) (Agilent Technologies Inc., Santa Clara, USA) and TOP10 (Life Technologies GmbH, Darmstadt, Germany) were cultivated at 37 °C in LB (lysogeny broth) medium. A. tumefaciens GV3101 ΔΔpc and derivatives at 30 °C in YEB (yeast extract broth) and Xcv strain 85-103 and derivatives on NYG (nutrient yeast glucose) agar plates supplemented with appropriate antibiotics. Plasmids were introduced into E. coli and A. tumefaciens by electroporation, and into Xcv by conjugation using pRK2013 as helper plasmid in triparental matings.

---

**Fig. 7** XopH causes InsP₅ accumulation in planta. **a** Upper panel: HPLC profiles of extracts from transgenic N. benthamiana leaves, labeled with [¹³C]-myo-inositol. Based on published chromatographic mobilities, InsP₅c represents InsP₅ [1-OH], InsP₅c represents InsP₅ [2-OH], InsP₅b represents InsP₅ [1/4/6-OH], and InsP₅c represents InsP₅ [1/3-OH]. The isomeric natures of InsP₅c, InsP₅b, InsP₅c, and InsP₅ are unknown. Middle panel: Zoom-in on the HPLC profiles. Lower panels: Relative amounts of InsP₅ and InsP₅c in N. benthamiana expressing gfp and two independent lines expressing XopH. Error bars indicate s.e.m. **b** Digestion and HPLC analyses of plant-purified InsP₅c species. InsP₅c and InsP₅ were purified from [¹³C]-myo-inositol-labeled xopH- and gfp-expressing N. benthamiana seedlings, respectively (see “Methods” section). XopH-treated or non-treated InsP₅ species (as designated) were then separated by SAX-HPLC. XopH was inactivated by incubating the reaction mixture at 95 °C for 15 min. **c** XopH hydrolyzes InsP₅ to InsP₅ during Xcv infection. TIO₂ bead enrichment of inositol polyphosphates from N. benthamiana leaf extracts infected with Xcv strains 85-10ΔxopQ and 85-10ΔxopQ,Fs-xopH, and the complemented mutant, where xopH was re-integrated into the genome (see “Methods” section). Inositol polyphosphates were eluted from TIO₂ beads, resolved by PAGE and visualized by toluidine blue staining. Protein extracts were visualized by Coomassie blue as a loading control. The experiments were repeated twice (a, c) or once (b) with similar results.
Fig. 8 XopH phytase activity affects plant hormone pathways. a qRT-PCR analysis of *N. benthamiana* leaves transiently expressing XopH and mutant derivatives, untreated or 20 min post wounding. ev empty T-DNA. Asterisks indicate statistically significant differences to corresponding ev samples (Mann–Whitney test; *p < 0.05; **p < 0.01; ***p < 0.001). b Protein expression two dpi in the same plants analyzed in a. Immunoblot signals at expected sizes are marked by asterisks. c qRT-PCR analysis of COI1-, EIN2-, and EBF1-silenced *N. benthamiana* plants (siCOI1, siEIN2, and siEBF1) transiently expressing XopH and its catalytically inactive variant (CH), respectively. Differences (Student’s t test; *p < 0.05; **p < 0.01) are shown (with three biological replicates per experiment) are shown (ch). OTS- silenced plants (siPfr1 mutant), and pENTR/D:xopH-mutP48-52-53-his6 (PRR1 mutant), pENTR/D:xopH-mutP69-71-his6 (PRR2-1 mutant), and pENTR/D:xopH-mutP73-74-75-76-his6 (PRR2-2 mutant) were derived from pENTR/D:xopH-his6, using the QuickChange Lightning Site-Directed Mutagenesis Kit (Agilent Technologies). To generate pENTR/D:xopH-C267A-his6, a 4-bp insertion after nucleotide (nt) position 53 of the ORF gen- 

### Construction of xopH mutants in *Xcv*. The *xopH* frameshift mutant (fs-*xopH*) carries a 4-bp insertion after nucleotide (nt) position 53 of the *xopH* ORF generating a P60 restriction site and resulting in an early stop codon at nt position 76. For this, plasmid pOK-early-stop-*xopH* was used containing two ~670-bp PCR 

### XopH derivatives and controls. Generally, DNA fragments were amplified using Hybrid DNA Polymerase (Roboklon, Berlin, Germany) unless stated otherwise. All cloned fragments were sequenced. Oligonucleotides are listed in Supplementary Table 1. Constructs were generated by GATEWAY® and In-Fusion HD (Takara Bio USA, Inc., Mountain View, USA) cloning technology according to the manu- 

### To generate expression constructs for XopH-His6, and XopH-Del2-77-His6, PCR-amplified *xopH* fragments were cloned into pENTR/D-TOPO (Thermo Fisher Scientific, Darmstadt, Germany). To generate pENTR/D:xopH-C267A-his6, the Phusion Site-Directed Mutagenesis Kit (Thermo Fisher Scientific) was used according to the manufacturer’s protocol. pENTR/D:xopH-H239A-his6, pENTR/D: 

### xopH-mutP48-52-53-his6 (PRR1 mutant), pENTR/D:xopH-mutP69-71-his6 (PRR2-1 mutant), and pENTR/D:xopH-mutP73-74-75-76-his6 (PRR2-2 mutant) were derived from pENTR/D:xopH-his6, using the QuickChange Lightning Site-Directed Mutagenesis Kit (Agilent Technologies). To generate pENTR/D:xopH-C267A-H239A-his6 (CH), pENTR/D:xopH-C267A-his6, served as template for site-directed mutagenesis. Entry clones were transferred into the GATEWAY-compatible expression vectors pAG416-GAL-ccdB-HA for yeast (gift from Susan Lindquist; Addgene plasmid #14243) and pGW2 for plants 47.

For localization studies, constructs for expression of GFP-XopH-His6 fusions with nuclear import (NLS), export (NES), or mutated export (nes) signal were generated by SOE (splicing by overlap extension) PCR using Q5 High Fidelity Polymerase (New England Biolabs, Frankfurt/Main, Germany), cloned into pENTR/D-TOPO and transferred into pGW2. As control, PCR-amplified gfp was cloned into pENTR/D-TOPO and transferred into pGW408 48. 

For expression in *E. coli*, PCR-amplified *xopH-his6*, and *xopH-Del2-77-his6*, fragments were cloned into NdeI/Xhol-digested pET22b(+) vector (Merck Millipore, Darmstadt, Germany) using In-Fusion HD cloning. 

For complementation, *xopH*, *xopHΔC*, and *xopHΔDelt277* respectively, under control of the native *xopH* promoter (344 bp) and followed by the avrB3 terminator region (208 bp) was inserted into a de-
Generation of transgenic xopH plants. N. benthamiana was transformed using Agrobacterium GV3101 containing pGW2:xopH-his, and pGW2:xopH H239A-C267A-his6, respectively. Transgenic plants were confirmed by PCR and immunoblot. Here, T2 plants were used.

Phosphopeptide microarray. The 13-mer peptide microarray used for profiling the XopH-phosphatase activity consists of 6267 peptides, each containing one phosphoryl-tyrosine residue in the central position. Each phosphopeptide is displayed in triplicates on glass surfaces enabling quality control and intra-chip reproducibility. Peptides were synthesized by SPOT synthesis technology on cellulose membranes according to R. Frank. After deprotection of side chains by 99% trifluoroacetic acid, spots were punched out into wells of 96-microtiter plates. Phosphopeptides were released from the cellulose membrane by treatment with 5% aqueous triethylamine. After separation of the peptide solution from the cellulose disks and evaporation of the cleavage solution peptide derivatives were redissolved in printing buffer and transferred into 384-well plates. Peptides were printed onto epoxy-functionalized glass slides using an OmniGrid3000 contact printer. Finally, microarray surfaces were passivated at 40 °C using bovine serum albumin in citrate buffer.

Protein microarrays reacted with XopH or the C267A variant yielded 82 and 238 substrate peptides, respectively, showing more than 70% signal decrease as compared to the control experiment without enzyme. Two types of control experiments were performed to distinguish between signal decrease caused by enzymatic action and signal decrease because of enzyme bound to the immobilized phosphopeptides and masking the epitope for the phospho-specific antibody (P100; Cell Signaling Technologies, Leiden, the Netherlands). First, peptide microarrays were treated with α-XopH antibodies and then the C267A variant did not bind to immobilized phosphopeptides under the conditions used. Thus, any epitope masking effect could be excluded.

Protein phosphatase assay on peptide microarrays. XopH and XopH<sub>C267A</sub> were incubated at room temperature (RT) with the peptide microarray using the TECAN Hybstation HS400. Prior to phosphatase treatment, two wash steps with phosphate-buffered saline (pH 7.5) with 0.1% Tween20 (PBST) and one wash step with PBS buffer were applied. Microarray blocking was performed with PBST and 3% bovine serum albumin (BSA) for 10 min, followed by 2× PBST and 1× PBS wash steps. Peptide microarrays were treated for 2 h with 10 μM <sup>32</sup>P-enzyme in reaction buffer (50 mM HEPES, pH 7.0, 100 mM NaCl, 1 mM dithiothreitol (DTT), 1% BSA, 1% Tween20, and 3% BSA). A control experiment without enzyme was done to obtain starting signal intensities for each spot. After washing with 5× PBST and 1× PBS buffer of ϕ-phospho-tyrosine mouse antibody (Cell Signaling Technology Europe, Leiden, the Netherlands) was applied at 10 μg mL<sup>−1</sup> concentration in PBST + 3% BSA for 1 h followed by 5× PBST and 1× PBST wash steps. For staining the phosphopeptide-bound antibody Dylight650-labeled anti-mouse IgG (Pierce, Thermo Fisher Scientific) was used (30 min at 1 μg mL<sup>−1</sup> in PBST, 3% BSA). Final washing steps were carried out with 5× PBST, 1× PBS, 2× distilled H<sub>2</sub>O and 2× nitrogen drying. To check for XopH binding to immobilized phosphopeptides, we applied ω-XopH-antibody (1:5000 diluted, 1 h), followed by 5× PBST and 1× PBST wash steps. For staining the phosphopeptide-bound antibody Alexa Fluor 594-labeled anti-ω-XopH-antibody (1:200 diluted, 30 min) and Alexa Fluor 594-labeled anti-α-XopH-antibody (1:5000 diluted, 1 h) was used to obtain starting signal intensities for each spot. After washing with 5× PBST, 1× PBS, 2× distilled H<sub>2</sub>O and 2× nitrogen drying. To determine if the signal decrease is caused by enzymatic action, we performed control experiments with inhibited phosphatase (0.1 mM sodium orthovanadate (Sigma–Aldrich)) or in the presence of excess of competing substrate (600 μM pTyr2 peptide (DADE[πLIP0QG]) using similar conditions for the microarray experiments. To remove bound phosphopeptide molecules from the immobilized phosphopeptides, we added two wash steps (20 min each) after XopH incubation (1 × 6 M urea and 1 × 3 M urea in dH<sub>2</sub>O, pH 7.4) followed by 3× dH<sub>2</sub>O wash steps. Fluorescence imaging of peptide microarrays was done using an Axon GenePix 4000B scanner at appropriate wavelengths, resolution 10 μm per pixel. Image evaluation was performed with GenePix Pro 7.2 (www.moleculardevices.com) followed by statistical analyses to identify top substrates and create two-sample logs (t test was chosen as statistical test and amino acid residue was shown if p value was below 0.05; http://www.twosamplelogo.org/cgi-bin/tst/tstl).

High-performance liquid chromatography kinetics. Phosphorylated peptides (pTyr2: Ac-DADE-pY LIPOQGW-NH<sub>2</sub> ϕ-Tyr-chip: Ac-KVVDY-pY- DENKFVV-NH<sub>2</sub> and negative control Ac-GKRKK-pY-KSLTRNW-NH<sub>2</sub>) were purchased from JPT Peptide Technologies (Berlin, Germany). Phosphopeptides were dissolved in 50 mM HEPES, pH 7.0, 10 mM NaCl, 1 mM DTT, 1 mM MgCl<sub>2</sub> at 0.5–50 μM at 37 °C and reaction was started by addition of 0.05 mg mL<sup>−1</sup> XopH. Reactions were stopped at different time points (1–120 min) by addition of 10% (v/v) trifluoroacetic acid (TFA) to a final concentration of 1% TFA (v/v). Reactions were analyzed using an Agilent 1100 series HPLC and Kineticz 2.6 μm XB-C18 100 A, 50 × 3.0 mm column for separation. Peak areas of phosphorylated and dephosphorylated peptides at different time points were used to determine the velocity of the reaction. Nonlinear least-squares curve-fitting according to the Michaelis-Menten model and calculation of K<sub>m</sub> and V<sub>max</sub> was performed using GraphPad Prism 5.01 software (www.graphpad.com).
**Chemical synthesis and analysis of pure InSp-P**

$^{1}H$-NMR spectra were recorded on a Bruker 400 MHz spectrometer at 298 K in the indicated deuterated solvent. Data are reported as follows: chemical shift (ppm), multiplicity (s, d, t, q, m, complex); J coupling constant(s) (Hz), unit). All signals were referenced to the internal solvent signal as standard (CDCl$_3$, 7.26; D$_2$O, 4.79; CD$_3$OD, 3.53; DMSO-d$_6$, 2.30).$^{31}$P-NMR spectra and $^{31}$P-NMR spectra were recorded with $^{1}H$-decoupling. $^{1}H$-NMR spectra were recorded on a Bruker 400 MHz spectrometer at 298 K in the indicated deuterated solvent. All signals were referenced to an internal standard (PPP). $^{13}$C[$^{1}H$]-NMR spectra were recorded with $^{1}H$-decoupling on a Bruker $^{1}H$-NMR spectra in DMSO-d$_6$ (298 K) with 2,4-dicyanoimidazole were added. Progress of the reaction was monitored by $^{31}$P-NMR. After completion of the reaction (30–45 min), the reaction mixture was diluted with D$_2$O to enable locking (total volume 600 µL). Residual XopH activity was destroyed by heating at 95 °C for 15 min. Spiking of the sample was performed with N. benthamiana seedlings grown in MS 2% solid sterile media were transferred into 3 mL liquid 0.5 MS, 1% sucrose, pH 5.7 liquid media supplemented with 100 µg mL$^{-1}$ Biotrend; ART-0261-5), then induced for 3 h with 1% PGR mixture and 0.25 M NaCl via the twisted PTFE coil (length 8 m; i.d. 0.5 mm) (HPLC pump 2248, Pharmacia; flow rate 0.8 mL min$^{-1}$). A series of InSp$_{P}$ standards were used to confirm the identity of the degradation products.

**Phytoase assay**

InSp$_{P}$ was purchased from Sichem (Bremen, Germany) or synthesized as outlined below. For initial experiments, also InSp$_{P}$ from Sigma-Aldrich was used. XopH$_{L}$-inositol pyrophosphatase was purchased from Sichem. Reaction mixtures (50 µL of 0.05 µg µL$^{-1}$ XopH$_{L}$-inositol. The seedlings were labeled for a total of six days. In order to label myo-inositol phosphates. The strain was generated by the deletion project (http://www-sequence.stanford.edu/group/-yeast_deletion_project/deletions3.html) and obtained from Open Biosystems (GE Healthcare). N. benthamiana transgenic lines were grown in glucose-free minimal media supplemented with 3% raffinose and 6 µCi mL$^{-1}$-[H]$^{3}$-myo-inositol (30–80 Ci µmol$^{-1}$); Biotrend; ART-0261-5), then induced for 3 h in minimal media containing 3% raffinose and 2% galactose. Cells were harvested, extracted, and analyzed by Partispher SAX HPLC$^{28}$. InSp$_{P}$-inositol polyphosphate analyses with extracts of N. benthamiana transgenic lines were done as follows. 10-day-old seedlings grown in MS 2% solid sterile media were transferred into 3 mL liquid 0.5 MS, 1% sucrose, pH 5.7 liquid media supplemented with 100 µg mL$^{-1}$-[H]$^{3}$-myo-inositol. The seedlings were labeled for a total of six days. In order to label adult leaves of N. benthamiana transgenic lines, leaves were then kept in 3 mL liquid 0.5 MS, 1% sucrose, pH 5.7 liquid media supplemented with 100 µg mL$^{-1}$-[H]$^{3}$-myo-inositol for 1 h at 28 °C. After labeling, the leaves were thoroughly washed in ultrapure water for two times before freeze harvesting into liquid N$_{2}$. InSp$_{P}$-inositol polyphosphates were extracted from the plant materials and resolved by SAX HPLC with the gradient of buffers A (1 mM EDTA) and B (1 mM EDTA and 1.3 M (NH$_4$)$_2$HPO$_4$, pH 3.8, with H$_3$PO$_4$). The experiments were repeated three times independently with similar results. Due to a change in the Partispher SAX column between runs, slight alterations in the elution times of inositol phosphates were observed.

**XopH digestion of HPLC-purified [H]$^{3}$-myo-inositol phosphates**

$^{[H]}$-InSp$_{P}$ and $^{[H]}$-InSp$_{P}$ were extracted and purified from SAX-HPLC runs of $^{[H]}$-myo-inositol-1,6-bisphosphate (Sigma-Aldrich) was purchased from Sigma-Aldrich. XopH activity is reported as release of inorganic phosphate (Pi) per minute (unit, a GAB gradient spectroscopy and 5 mm BB observe probe (1H; 19F–109Ag: 564.7–279.54 MHz). The samples were heated at 300 K during the measurements (NS: 8–40, A: 0.34; DW: 5.2; slice thickness: 1 mm). XopH activity was assessed by heating to 95 °C for 15 min. Spiking of the sample was performed with commercial InSp$_{P}$ in different ratios (1:5, 1:1, and 1:1.5) in the presence of L-Arg-N (100-150-fold excess, adjusted during the spiking experiments).

**Phytoaspartate assay**

Phytoaspartate was purified from Bradyrhizobium japonicum by a two-step procedure. In a 15 mL reaction buffer composed of 50 mM HEPES (pH 7.0), 10 mM NaCl, 5% glycerol and 0.1% β-mercaptoethanol. XopH reaction products were separated using PAGE and purified by two dehydration–hydration cycles$^{48}$. Unprocessed gel images are provided in Supplementary Fig. 13.
and aliquoted to 130 mg each for further analysis. One aliquot was used for protein analysis of each strain were harvested and frozen in liquid nitrogen. The samples were pooled and analyzed by SDS-PAGE.

Thamiana

4. Teper, D., Sunitha, S., Martin, G. B. & Sessa, G. Five recombinant XopH enzyme assays, 20 nmol InsP6 were incubated with XopH for 1–2 min at 28 °C. Non-targeted ion detection by LC-MS was achieved using an Acquity UPLC Waters) and a TripleTOF 5600 mass spectrometer using the software Analyst 1.6 TF (Sciex, Toronto, Canada). Inositol polyphosphates were separated on a Nucleosil RP18 (150 mm × 2 mm × 7.8 μm, Macherey & Nagel, Düren, Germany) by ion pairing chromatography using solvent A: 10 mM tributyl amine (Sigma-Aldrich), which was acidified with glacial acetic acid to pH 6.2 and solvent B: acetonitrile. Gradient (% B): 0–2 min: 2, 18 min: 5, 21.5 min: 95, 22.52 min: 95, 22.54 min: 2, with a column flow of 400 μL/min and a column temperature of 40 °C.

Electrospray ionization was achieved in negative mode using a DuoSource3M (Sciex, Toronto, Canada). Source temperature: 600 °C, ion spray voltage: –4500 V, spray gas: 35 psi, source gases ∶/∶ 60/40 psi. TOF-MS mass features were assayed between 65 and 1250 Da simultaneously an array of non-targeted QToF-MS scan experiments in SWATH mode. During the latter, the transmission range for precursor ions in the Q1-quadrupol was set for 20 ms to mass windows of 33 Da and was incremented from m/z 65–1250 Da. The declustering potential was kept at +35 V, the collision energy was ramped from +10 –45, and –60 V using the collision energy spread option. Mass accuracy (below 5 ppm) was automatically re-calibrated every 20th measurement using the calibrant delivery system, APCI ionization (Duo-Source3M) and the APCI calibrator mixture (all Sciex, Toronto, Canada). Simultaneous MS1–TOF scanning and SWATH-CID-fragmentation allowed for rapid assessment of MS3 and MS5 spectra with cycle times of 1 s. The measurements were performed twice with independent samples giving similar results.

Data availability. The authors declare that all relevant data supporting the findings of this study are available within the paper and its supplementary information files or are available from the corresponding authors on request.

References

1. Böttner, D. Behind the lines-actions of bacterial type III effector proteins in plant cells. FEMS Microbiol. Rev. 40, 894–937 (2016).
2. Jones, J. B., Lacy, G. H., Bouzari, H., Stall, R. E. & Schaad, N. W. Reclassification of the xanthomonads associated with bacterial spot disease of tomato and pepper. Syst. Appl. Microbiol. 27, 755–762 (2004).
3. Thieme, F. et al. Insights into genome plasticity and pathogenicity of the plant pathogenic bacterium Xanthomonas campestris pv. vesicatoria revealed by the complete genome sequence. J. Bacterial. 187, 754–776 (2005).
4. Teper, D., Sunitha, S., Martin, G. B. & Sessa, G. Five Xanthomonas type III effectors suppress cell death induced by components of immunity-associated MAP kinase cascades. Plant Signal. Behav. 10, e1064573 (2015).
5. Mur, L. A., Kenton, P., Lloyd, A. J., Ougham, H. & Prats, E. The hypersensitive response; the centenary is upon us but how much do we know? J. Exp. Bot. 59, 501–520 (2008).
6. Boch, J. & Bonas, U. Xanthomonas AvrBs3 family-type III effectors: discovery and function. Annu. Rev. Phytopathol. 48, 419–436 (2010).
7. Singer, A. U. et al. A pathogen type III effector with a novel E3 ubiquitin ligase architecture. PLoS Path. 9, e1003121 (2013).
8. Kibra, M. S. et al. AvrRToT activates the Arabidopsis ACIP1, a protein that associates with microtubules and is required for immunity. PLoS Path. 10, e1003952 (2014).
9. Potnis, N. et al. Avirulence proteins AvrB67 from Xanthomonas gardneri and AvrB61 from Xanthomonas euvesicatoria contribute to a novel gene–gene interaction in pepper. Mol. Plant Microbe Interact. 25, 307–320 (2012).
10. Shimomura, L., Arescues, E., Y. & Szedlacek, S. E. Protein tyrosine phosphatases: structure-function relationships. FEBS J. 275, 867–882 (2008).
11. Kay, B. K., Williamson, M. P. & Sudol, M. The importance of being proline: the interaction of proline-rich motifs in signaling proteins with their cognate domains. FASEB J. 14, 231–241 (2000).
12. Lim, K. L., Lin, D. S. Y., Kalousek, M. B., Wang, Y. & Pallen, C. J. Kinetic analysis of two closely related receptor-like protein-tyrosine phosphatases, PTPalpha and PTPepson. Eur. J. Biochem. 245, 693–700 (1997).
13. Zhang, Z. Y., Maclean, D., Thiemesfedler, A. M., Roeseke, R. W. & Dixon, J. E. A continuous spectrophotometric and fluorometric assay for protein-tyrosine phosphatase using phosphotyrosine-containing peptides. Anal. Biochem. 211, 251–255 (1993).
14. Kelley, L. A., Mezulis, S., Yates, C. M., Wassenberg, M. N. & Sternberg, M. J. The Phe2y web portal for protein modeling, prediction and analysis. Nat. Protoc. 10, 845–858 (2015).
15. Chu, H.-M. et al. Structures of Selenomonas ruminantium phytase in complex with persulfated phytate: DSP phytase fold and mechanism for sequential substrate hydrolysis. Structure 12, 203–204 (2004).
16. Rabyo, V., myo-Inositol-1,2,3,4,5,6-hexakisphosphate. Phytochemistry 64, 1033–1043 (2003).
17. Murphy, A. M., Otto, B., Brearley, C. A., Carr, J. P. & Hanke, D. E. A role for inositol hexaphosphate in the maintenance of basal resistance to plant pathogens. Plant J. 56, 638–652 (2006).
18. Laha, D. et al. Inositol polyphosphate binding specificity of the jasmonate receptor complex. Plant Physiol. 171, 2364–2370 (2016).
19. Popov, G., Fraiture, M., Brunner, B. & Sessa, G. Multiple Xanthomonas euvesicatoria type III effectors inhibit flg22-triggered immunity. Mol. Plant Microbe Interact. 29, 651–660 (2016).
20. Greiner, R. & Konietzny, U. In Enzymes in Farm Animal Nutrition 2nd edn, (eds Bedford, M. & Patridge, G.) 96–128 (CAB International, Wallingford, UK, 2010).
21. Konietzny, U. & Greiner, R. Molecular and catalytic properties of phytate-degrading enzymes (phytases). Int. J. Food Sci. Technol. 37, 791–812 (2002).
22. Puhl, A. A. et al. Kinetic and structural analysis of a bacterial protein tyrosine phosphatase-like myo-inositol polyphosphatase. Protein Sci. 16, 1366–1378 (2007).
23. Yang, L. et al. Expedited selection of NMR chiral solvating agents for determination of enantiopurity. ACS Cent. Sci. 2, 332–340 (2016).
24. Li, Y. & Raushel, F. M. Differentiation of chiral phosphorus enantiomers by 31P and 31H NMR spectroscopy using amino acid derivatives as chemical solvating agents. Tetrahedron 18, 1391–1397 (2007).
25. Moon, R. B. & Richards, J. H. Determination of intracellular pH by 31P magnetic resonance. J. Biol. Chem. 248, 7276–7278 (1973).
26. Adlung, N. et al. Non-host resistance induced by the Xanthomonas effector XopQ is widespread within the genus Nicotiana and functionally depends on EDS1. Front. Plant Sci. 7, 1796 (2016).
27. Sheard, L. B. et al. Jasmonate perception by inositol-phosphate-potentioted COI1-JAZ co-receptor. Nature 468, 400–405 (2010).
28. Tan, X., Calderon-Villalobos, L. I. A., Sharon, M. & Zheng, C. Mechanism of auxin perception by the TIR1 ubiquitin ligase. Nature 446, 640–645 (2007).
29. Laha, D. et al. VIH2 regulates the synthesis of inositol phosphatase InsP6 and jasmonate-dependent defenses in Arabidopsis. Plant Cell 27, 1082–1097 (2015).
30. Shoji, T. & Hashimoto, T. Tobacco MYC2 regulates jasmonate-inducible nicotine biosynthesis genes directly and by way of the NIC2-locus ERF genes. Plant Cell Physiol. 52, 1117–1130 (2011).
31. Kiba, A. et al. SECI4 phosphotransfer protein is involved in lipid signaling-mediated plant immune responses in Nicotiana benthamiana. Plant Cell 32, 5809–5826 (2015).
32. Adie, B., Chico, J., Rubio-Somoza, I. & Solano, R. Modulation of plant defenses by ethylene. J. Plant Growth Regul. 26, 160–177 (2007).
33. Donnell, P., Calvert, C., Atzorn, R. & Wasternack, C. Ethylene as a signal mediating the wound response of tomato plants. Science 274, 1914–1917 (1996).
34. Zhu, Z. & Lee, B. Friends or foes: new insights in jasmonate and ethylene co-actions. Plant Cell Physiol. 56, 414–420 (2015).
35. Xie, D. X., Feys, B. F., James, S., Nieto-Rostro, M. & Turner, J. G. COI1: an Arabidopsis gene required for jasmonate-regulated defense and fertility. Science 280, 1091–1094 (1998).
36. Macho, A. P. et al. A bacterial tyrosine phosphatase inhibits plant pattern recognition receptor activation. Science 343, 1509–1512 (2014).
37. Chatterjee, S., Sankaranarayanan, R. & Sonti, R. V. PhyA, a secreted protein of Xanthomonas oryzae pv. oryzae, is required for optimum virulence and growth on phytic acid as a sole phosphate source. Mol. Plant Microbe Interact. 16, 973–982 (2003).
38. Lund, S. T., Stall, R. E. & Klee, H. J. Ethylene regulates the susceptible response to pathogen infection in tomato. Plant Cell 10, 371–382 (1998).
39. Weber, S. E. et al. A type IV translocated Legionella cytostere phytase counteracts intracellular growth restriction by phytate. J. Biol. Chem. 289, 34175–34188 (2014).
40. Stephens, L. R. et al. myo-inositol pentakisphosphates. Structure, biological occurrence and phosphorylation to myo-inositol hexakisphosphate. Biochem. J. 275, 485–499 (1991).
41. Bertani, G. Studies on lysogenesis. I. The mode of phage liberation by lysogenic cells for protein analysis. J. Biol. Chem. 261, 293–300 (1986).
42. Van Larebeke, N. et al. Large plasmid in Agrobacterium tumefaciens essential for crown gall-inducing ability. Nature 252, 169–170 (1974).
43. Daniels, M. J. et al. Cloning of genes involved in pathogenicity of Xanthomonas campestris pv. campestris using the broad host range cosmid pLAFR1. EMBO J. 3, 3323–3328 (1984).
44. Figurski, D. H. & Helmann, D. R. Replication of an origin-containing derivative of plasmid RK2 is dependent on a plasmid function provided in trans. Proc. Natl Acad. Sci. USA 76, 1648–1652 (1979).
45. Huguet, E., Hahn, K., Wengelnik, K. & Bonas, U. hpa mutants of Xanthomonas campestris pv. vesicatoria are affected in pathogenicity but retain the ability to induce host-specific hypersensitive reaction. Mol. Microbiol. 29, 1379–1390 (1998).
46. Walhout, A. J. M. et al. GATEWAY recombinational cloning: application to the cloning of large numbers of open reading frames or ORFeomes. Method Enzymol. 328, 575–592 (2000).
47. Nakagawa, T. et al. Development of series of gateway binary vectors, pGWBs, for realizing efficient construction of fusion genes for plant transformation. J. Biosci. Bioeng. 104, 34–41 (2007).
48. Nakagawa, T., Ishiguro, S. & Kimura, T. Gateway vectors for plant research. EMBO J. 19, 4150–4153 (2000).
49. Lorence, P. A., Henning, P. Johnen, C. Kretschmer, C. Liechti, N. F. Rebollo, and B. Rosinsky for technical assistance. This work was funded by grants from the Bundesministerium für Bildung und Forschung (“Tools, targets & therapeutics – ProNet T3 to U.B. and J.L.”), the Deutsche Forschungsgemeinschaft (SFB 648/TP A1 and B1 to U.B. and J.L., Gottfried Wilhelm Leibniz Prize to U.B., SFB 1101/TP A05 and research grant CHA 1274/4/1 to G.S.), the Swiss National Science Foundation (PP00P2_157607 to H.J.), and the Carl-Zeiss Stiftung (to H.J.). Publication was supported by the Open Access Publication Fund of the Martin-Luther-Universität Halle-Wittenberg. We thank the NMR facilities of UZH and Uni Freiburg (Magres) for extended measurement time.

Additional information
Supplementary Information accompanies this paper at https://doi.org/10.1038/s41467-017-02195-8.

Competing interests: The authors declare no competing financial interests.

Reprints and permissions information is available online at http://npg.nature.com/reprintsandpermissions/

Publisher’s note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in a credit line to the material. If not included in the article’s Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/.

© The Author(s) 2017