A bacterial kinase phosphorylates OSK1 to suppress stomatal immunity in rice

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The Xanthomonas outer protein C2 (XopC2) family of bacterial effectors is widely found in plant pathogens and Legionella species. However, the biochemical activity and host targets of these effectors remain enigmatic. Here we show that ectopic expression of XopC2 promotes jasmonate signaling and stomatal opening in transgenic rice plants, which are more susceptible to Xanthomonas oryzae pv. oryzicola infection. Guided by these phenotypes, we discover that XopC2 represents a family of atypical kinases that specifically phosphorylate OSK1, a universal adaptor protein of the Skp1-Cullin-F-box ubiquitin ligase complexes. Intriguingly, OSK1 phosphorylation at Ser53 by XopC2 exclusively increases the binding affinity of OSK1 to the jasmonate receptor OsCOI1b, and specifically enhances the ubiquitination and degradation of JAZ transcription repressors and plant disease susceptibility through inhibiting stomatal immunity. These results define XopC2 as a prototypic member of a family of pathogenic effector kinases and highlight a smart molecular mechanism to activate jasmonate signaling.

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Any phytopathogenic bacteria inject effector proteins into host cells through the type III secretion system to inhibit plant defenses for successful infection.1-3. The intense evolutionary arms race between the pathogen and its host, together with inter-kingdom horizontal gene transfer, generates remarkable sequence diversity of pathogen effectors,4 which makes it difficult to elucidate the biochemical functions of many pathogen effectors.

Elegant biochemical analyses combined with improved bioinformatics methods can facilitate the identification of novel functions associated with pathogen effectors, which provide insights into not only new pathogenicity mechanisms but also previously unrecognized aspects of host cell biology and signaling pathways.5 For example, PSI-BLAST analysis revealed a putative mono-ADP-riboseyltransferase motif in the central region of members of the SidE effector family from *Legionella pneumophila*, which was subsequently found to catalyze a novel E1/E2-independent ubiquitination reaction.6 The *Xanthomonas campestris* pv. *campestris* type III effector AvrRac was identified as a uridylyltransferase that modifies plant BIK1 and RIPK kinases, and consequently inhibits their kinase activity and downstream signaling.7 The *Pseudomonas syringae* effector HopBF1 has been demonstrated to function as an atypical kinase that attacks the HSP90 chaperone of host cells.8 Several other effector families have also been identified as protein kinases that target diverse host cellular processes.9-14. These characterized effector kinases mainly belong to two classes. Class I effectors such as YpkA, XopAU, SteC, and LegK1 show a high sequence and structure similarity to eukaryotic kinase12,15, and class II effectors exemplified by NleH1, OspG, and HopBF1 harbor only basic kinase motifs and have lost several conserved subdomains found in canonical kinases9,13,14.

*Xanthomonas*-spp. cause many important diseases in a variety of plant species. *X. oryzae*, *X. campestris*, and *X. axonopodis* pathovars, for example, are among the top 10 important plant pathogenic bacteria.16 *Xanthomonas*-spp. secrete two classes of type III effectors, transcription-activator-like (TAL) and non-TAL effectors, into host cells.17 The TAL effectors are usually translated into host cell nuclei and function as a unique family of transcription activators.18 For example, *X. oryzae* PthXo1 activates the transcription of membrane-bound sugar transporter gene OsSN3, resulting in pumping out intracellular sugars into apoplastic spaces to feed bacteria19,20. AvrBs3 in *X. campestris* pv. *vesicatoria* targets a cell size regulator gene *upa20* to induce hypertrophy of plant mesophyll cells to promote infection.21

The non-TAL effectors also play important roles in bacterial infection and disease development.22 XopD is an active Ulp1-like cysteine protease that suppresses plant immunity by catalyzing the deSUMOylation and destabilization of transcription factor SIERF4 in tomato.23,24 XopH is a 1-phytase that dephosphorylates myo-inositol-hexakisphosphate (InsP6) to generate InsP5 and interferes with plant hormone signaling.25 Furthermore, several *Xanthomonas* effectors including XopK, XopL, and XopAE represent different types of ubiquitin E3 ligases.26-28 Interestingly, XopAI/AvrRxo1 functions as a NAD kinase, which phosphorylates NAD to produce 3’-NADP and thus suppresses ROS burst.29,30 As a conventional serine/threonine kinase, XopAU in *X. euvesicatoria* manipulates MAPK signaling by phosphorylation and activation of MKK2.12 Despite significant progress, the molecular mechanisms of the functions of most effector proteins in phytopathogenic bacteria remain unknown.

*X. oryzae* pv. *oryzicola* (Xoc) infects rice leaves through stomata and wounds and causes bacterial leaf streak, one of the most important bacterial diseases in rice.31 Stomatal immunity greatly restricts bacterial infection at the very early infection stage.32 As a countermeasure, phytopathogenic bacteria secrete effector proteins and phytoxins to suppress stomatal immunity. For instance, *P. syringae* generates the JA-mimicking phytoxin coronatine and effector proteins, such as HopZ1 and HopX1, to activate JA signaling, thereby suppressing stomatal closure to facilitate bacterial entry of host tissues.33-35 Several non-TAL effector genes, such as avrBs2, *hrpE3*, and *Xrp5*, are required for full virulence of *Xoc*.36-37 However, little is known on the molecular mechanisms of how these effectors promote Xoc infection. In this study, we report that XopC2 in *Xoc* represents a family of core non-TAL type III effectors in *Xanthomonads*.38 XopC2 homologs are widely distributed in other pathogenic bacterial species, such as *Acidovorax* and *Ralstonia* spp. We demonstrate that XopC2 functions as a novel type of kinase that phosphorylates OsK1, a universal adaptor protein of SCF complex, at Ser13 residue. The phosphorylation of OsK1 at the specific site enhances the recruitment of OsCOI1b to the SCF complex and activates JA signaling.

**Results**

**XopC2 defines a novel family of bacterial effector kinases.** PSI-BLAST analysis uncovered that XopC2 has homologs with high-level similarity in a wide range of phytopathogenic bacteria, including *A. citrulli* and *R. solanacearum*, and even in *Legionella* species (Supplementary Fig. 1). No known structural or functional domain was predicted in XopC2 and its homologs via SMART, Pfam, and Phyre2 searches. However, a region in the carboxyl portion encompassing 391 to 417 amino-acid residues is highly conserved in these proteins revealed by sequence alignment and is predicted as a putative catalytic motif of protein kinases using HHpred (Fig. 1a and Supplementary Fig. 1). In addition, a P-loop-like motif featured with glycine-rich sequences and conserved lysine-serine/threonine (K/[S/T]) residues at the N-terminus might serve as a phosphate-binding motif (Supplementary Fig. 1). The conserved Lys147, Asp391, and Asp413 residues of XopC2 are predicted to be the catalytic triad and the Asn396 residue most likely coordinates the second Mg2+ ion and is involved in phosphoryl transfer.39 (Fig. 1a, b and Supplementary Fig. 1). These characteristics prompted us to investigate whether XopC2 might function as a protein kinase via in vitro kinase assays. Indeed, purified XopC2 exhibited autophosphorylation (Fig. 1c). The mutated XopC2 proteins with Asp391 and Asn396 residues replaced with Ala had a significantly reduced autophosphorylation activity (Fig. 1c). These results indicate that XopC2 is a functional protein kinase. Although XopC2 is not matched with any identified protein kinase from primary sequence alignment, the predicted secondary structure of XopC2 shows a similar topology to the known protein kinase A (PKA). By contrast, XopC2 contains more α-helix subdomains in two central regions, one between subdomains III and IV and the other between subdomains V and VI (Fig. 1b). The extra subdomains make the kinase domain of XopC2 (~470 amino acids) much longer than that of canonical kinases (250-300 amino acids). Phylogenetic analyses showed that XopC2 homologs form an independent cluster separated from any known protein kinase family (Supplementary Fig. 2). Thus, XopC2 represents the prototypic member of a family of protein kinases.

**XopC2-expressing rice plants exhibit increased disease susceptibility.** To investigate virulence functions of XopC2, 31 transgenic rice lines with constitutive expression of XopC2 driven by the CaMV 35S promoter (OE lines hereinafter) and 21, 24, and 27 transgenic lines with induced expression of XopC2 and the mutant variants XopC2-D931A and XopC2-N396A under dexamethasone (DEX)-inducible promoter (IE lines hereinafter), respectively, were generated through *Agrobacterium*-mediated
transformation. Expression of XopC2-FLAG and its variants in these transgenic lines was detected by immunoblotting (Supplementary Fig. 3a, b). As compared with the wild-type plants, the independent homozygous OE-1, OE-10, IE-17, and IE-37 transgenic lines exhibited no alteration in growth and agronomic traits, including seedling and plant heights, leaf width, chlorophyll content, and hundred-grain weight (Supplementary Fig. 3).

After pressure infiltration with the wild-type Xoc RS105 and ΔxopC2 mutant strains, the IE-17 transgenic line exhibited no significant difference in disease susceptibility regardless of mock and DEX treatments (Supplementary Fig. 4a). By contrast, the DEX-treated IE-17 transgenic line exhibited more disease lesions than the wild-type and mock-treated transgenic rice plants after spray inoculation with the Xoc ΔxopC2 strain (Supplementary Fig. 4b). Consistent with disease symptoms, in planta bacterial population in the DEX-treated IE-17 transgenic line was significantly greater than those in the wild-type and mock-treated transgenic lines (Fig. 2a; Supplementary Fig. 4c). However, the DEX-treated IE-D391A-2 and IE-N396A-14 transgenic lines had equal in planta bacterial population sizes to the wild-type and mock-treated transgenic plants (Fig. 2a). Collectively, these results suggest that XopC2 contributes to the initial steps of natural infection of Xoc in a kinase activity-dependent way.

In plants, stomatal immunity prevents phytopathogenic bacteria from entering host tissues as a barrier to initial infection. To investigate whether XopC2 disarms stomatal immunity, stomatal conductance (Gs) was measured for rice leaves after spray inoculation with ΔxopC2. The DEX-treated IE-17 and IE-37 transgenic lines had significantly higher Gs than the wild-type and mock-treated IE lines after ΔxopC2 inoculation, indicating that DEX-induced XopC2 expression compromises stomatal closure (Fig. 2b; Supplementary Fig. 4d). The wild-type, IE-D391A-2, and IE-N396A-14 seedlings showed no significant difference in Gs regardless of mock and DEX treatments, although DEX-treated IE-N396A-14 seedlings had a slightly higher Gs than mock-treated seedlings after ΔxopC2 infection (Fig. 2b). In addition, time course assays showed that the Gs of rice leaves was gradually decreased after Xoc inoculation, while the wild-type and OE-1 and OE-10 lines showed higher Gs at 24 h after RS105 and C-ΔxopC2 inoculation and thereafter (Supplementary Fig. 4e). Furthermore, the OE-1 and OE-10 lines showed higher Gs than the wild-type plants after ΔxopC2 inoculation, while the wild-type and OE transgenic lines had similar Gs after RS105 inoculation (Supplementary Fig. 4f). These data indicate that XopC2, but not the catalytic mutants, suppresses stomatal closure triggered by Xoc infection.

XopC2 promotes OsJAZ degradation and JA signaling. Stomatal defense is often compromised when jasmonic acid (JA) signaling is activated in the context of pathogen infections. To investigate this possibility, the expression of the JA-responsive marker genes including OsLOX2 and OsJAZ8 was analyzed in the xopC2 transgenic plants. OsLOX2 and OsJAZ8 expression was induced in the wild-type plants by exogenous methyl jasmonate (MeJA), but not by DEX treatment. By contrast, MeJA-induced expression of OsLOX2 and OsJAZ8 in IE-17 and IE-37 transgenic seedlings was significantly enhanced after DEX treatment compared with that in mock-treated transgenic seedlings (Fig. 3a, b; Supplementary Fig. 5a, b). However, MeJA-induced expression of OsLOX2 and OsJAZ8 was no longer enhanced in the DEX-treated IE-D391A-2 line, while was significantly promoted in the DEX-induced IE-N396A-14 line. Besides, JA-induced leaf senescence in the IE-17 and IE-37 lines was also promoted by DEX treatment.
compared with mock treatment (Supplementary Fig. 5c). In contrast, the SA and JA contents were not significantly different in mock-treated and DEX-treated wild-type and IE-17 lines (Supplementary Fig. 5d, e). In addition, either expression of OsICS1 and OsPAL1 (the SA-biosynthesis genes) or induced expression of OsAA9 and D10 by auxin and strigolactone, respectively, was not altered in the IE transgenic lines after DEX treatment (Supplementary Fig. 5f–i). These results indicate that ectopically expressed XopC2 specifically promotes JA signaling in rice.

Jasmonate activates JA signaling by promoting JAZ degradation. To investigate whether XopC2 induces degradation of OsJAZ proteins, HA-tagged OsJAZ7, OsJAZ9, OsJAZ12, and OsJAZ13 proteins were transiently expressed in rice protoplasts isolated from xopC2 transgenic seedlings. The accumulation of all examined OsJAZs-HA was dramatically reduced in the DEX-treated transfected protoplasts compared with those in mock-treated ones as revealed by immunoblotting (Fig. 3c and Supplementary Fig. 6a). OsJAZ9-HA accumulation was not altered by DEX treatment per se when the protein was transiently expressed in the protoplasts prepared from wild-type seedlings (Supplementary Fig. 6b). In addition, MG132, a proteasome inhibitor, largely prevented XopC2-promoted OsJAZ degradation (Fig. 3c and Supplementary Fig. 6a). Collectively, these findings indicate that XopC2 promotes the 26S proteasome-mediated degradation of OsJAZs.

To examine OsJAZ degradation during Xoc infection, we generated the OsJAZ9-HA-NE and OsJAZ9-HA-OE transgenic rice plants expressing OsJAZ9-HA driven by the native and CaMV 35S promoters, respectively, (Supplementary Fig. 7a) and determined the stability of OsJAZ9-HA in these plants after inoculation of the wild-type, ΔxopC2 or complemented Xoc strains. Spray inoculation of the wild-type strain caused evident degradation of OsJAZ9-HA in these transgenic seedlings, while OsJAZ9-HA was stable in the ΔxopC2-inoculated transgenic seedlings (Fig. 3d and Supplementary Fig. 7b). Interestingly, the plasmid-borne xopC2 gene restored the ability of the C-ΔxopC2 complementation strain to promote OsJAZ9-HA degradation during infection, whereas the mutated xopC2 gene encoding XopC2D391A did not (Fig. 3d and Supplementary Fig. 7b). Collectively, these results indicate that XopC2 secreted by Xoc promotes JAZ protein degradation during infection.

XopC2 enhances OsJAZ9 ubiquitination in vitro. JAZ proteins are ubiquitylated by the Skp1-Cullin-F-box type E3 ubiquitin ligase complex SCFCO1 and are then subjected to degradation via the 26S proteasome. To investigate whether XopC2 enhances ubiquitination of OsJAZ proteins, a semi-in vitro ubiquitination assay was performed. In this assay, His6-OsJAZ9 was first expressed in E. coli and then bound to nickel-agarose beads. His6-OsJAZ9-bound beads were then incubated with HA-ubiquitin and total cell extracts isolated from rice seedlings in the ATP-containing ubiquitination buffer. Immunoblotting with anti-HA-HRP showed that His6-OsJAZ9 bound to beads was ubiquitylated, while no ubiquitination signal was detected in the absence of total rice protein extracts (Fig. 3e). The ubiquitination of His6-OsJAZ9 was enhanced when GST-XopC2 was included in the reaction (Fig. 3e).

We next refined the semi-in vitro ubiquitination assay. The SCFCo1b complex was immunoprecipitated from rice protoplasts expressing OsCo1b-FLAG with anti-FLAG M2 affinity gel and then incubated with E1, E2, ubiquitin, and His6-OsJAZ9 for ubiquitination assays. This assay showed that OsJAZ9 ubiquitination was significantly enhanced in the presence of GST-XopC2, whereas both GST-XopC2D391A and GST-XopC2N396A largely lost the ability to enhance His6-OsJAZ9 ubiquitination (Fig. 3f). The results suggest that the kinase activity of XopC2 is critical for its ability to promote JAZ ubiquitination.

XopC2 interacts with and phosphorylates the adaptor protein OSK1 in SCFCo1b. To elucidate how XopC2, as a protein kinase, promotes JAZ ubiquitination, we investigated whether XopC2 phosphorylates specific component(s) of the SCFCo1b complex.
In vitro kinase assays showed that OSK1, but not other components including OsJAZ9, HA-ubiquitin, OsUBA1 (E1), UBCH5α (E2), OsCullin1α, OsRBX1, or OsCOI1b, was phosphorylated by XopC2 (Fig. 4a and Supplementary Fig. 8a). Subsequently, liquid chromatography-tandem mass spectrometry (LC-MS/MS) revealed that three amino-acid residues including Thr32, Ser53, and Ser92 in OSK1 were phosphorylated by XopC2 in vitro (Supplementary Fig. 8b). Based on structural modeling, the three residues together with seven other residues including Ser15, Thr62, Ser83, Thr133, Thr142, Thr149, and Thr157 are predicted to reside...
Fig. 3 XopC2 enhances JA signaling by promoting ubiquitination and degradation of JAZ proteins. a, b MeJA-induced expression of the JA-responsive genes OsLOX2 (a) and OsJAZ8 (b) was enhanced in XopC2-expressing rice seedlings. The wild-type, IE-17, IE-D391A-2, and IE-N396A-14 transgenic plants were treated with DEX or mock solution for 24 h followed by MeJA application (50 μM). Gene expression was detected by qRT-PCR using OsActin as an internal reference gene. Data are presented as means ± SE (n = 3 replicates per measurement). Asterisk (*) indicates a statistically significant difference in relative gene expression between mock and DEX treatments (two-sided t-test; * P < 0.05; ** P < 0.01; *** P < 0.001). c The accumulation of OsJAZ8s-HA was greatly reduced in rice protoplasts conditionally expressing XopC2-FLAG. OsJAZ7-HA and OsJAZ8-HA were transiently expressed in IE-17 transgenic rice protoplasts after mock and DEX treatments for 12 h. Western blotting was performed to detect OsJAZs-HA, XopC2-FLAG, and β-Actin (as a protein loading control). MG312, a proteasome inhibitor. d OsJAZ9-HA was rapidly degraded during Xoc infection but remained relatively stable during XopC2 infection. Three-week-old transgenic seedlings expressing OsJAZ9-HA driven by its native promoter were sprayed with the wild-type, XopC2, and XopC2 strains complemented with a wild-type copy of xopC2 (C-xopC2) or a kinase-defective copy (C-ΔxopC2Δ399P). OsJAZ9-HA was detected by immunoblotting at the indicated timepoints post-inoculation. e OsJAZ9 ubiquitination was enhanced in the presence of XopC2 in a semi-in vitro assay. After incubation with His6-OsJAZ9, NTAs beads were divided equally and were mixed with GST-XopC2, HA-ubiquitin (Ub), and total rice protein extracts (TRPE) as indicated in the ubiquitination buffer. The protein loading was shown by CBB staining. f OsJAZ9 ubiquitination was enhanced in the presence of GST-XopC2 revealed by a refined semi-in vitro assay. The SCFCOI1b complex was immunoprecipitated with anti-FLAG M2 affinity beads from the extract of rice protoplasts co-expressing OsCOI1b-FLAG, OsCullin1a-HA, OSK1-HA, and OsRbx1-1-HA. The SCFCOI1b complex was then incubated with human UBE1 (E1), UBC12-HA, HA-ubiquitin (Ub), His6-OsJAZ9, coronatine (COR), and GST-XopC2/XopC2D391A/XopC2N396A in the ATP-containing reaction buffer. OsJAZ9 ubiquitination was detected by immunoblotting with anti-HA-HRP and anti-His-HRP antibodies. The experiments were independently repeated 3 times with similar results in the panels a-f.

on the surface of OSK1 3-D structure and are candidate phosphosites (Supplementary Fig. 8c). To confirm the phosphorylation residues in OSK1 by XopC2, ten OSK1 variants with individual residues mutated to alanine were generated. The OSK1T32A, OSK1S53A, OSK1S92A, and OSK1T149A variant proteins exhibited significantly reduced phosphorylation when they were incubated with XopC2 in vitro in kinase assays (Supplementary Fig. 8d). These results indicate that these four residues are likely the major OSK1 phosphorylation sites by XopC2.

To detect whether XopC2 phosphorylates OSK1 during Xoc infection, we generated the transgenic rice plants expressing OSK1-FLAG (Supplementary Fig. 9a). The transgenic plants were inoculated with the wild-type, ΔxopC2, and xopC2-complemented strains. Total cell extracts were isolated from the inoculated leaves at 48 h after inoculation and were immunoprecipitated by anti-FLAG beads. Immunoblotting with an anti-pSer antibody revealed that serine phosphorylation of OSK1-FLAG was significantly enhanced in Xoc-inoculated plants compared with that in mock-treated and ΔxopC2-inoculated plants (Fig. 4b). Furthermore, inoculation of the xopC2-complemented strain activated OSK1 phosphorylation in planta, while the xopC2D391A-transformed strain did not (Fig. 4b). These results indicate that OSK1 phosphorylation is enhanced by XopC2 during Xoc infection. Interestingly, immunoprecipitated XopC2-FLAG from rice protoplasts showed a much higher ability to phosphorylate His6-OSK1 than XopC2 purified from E. coli, whereas XopC2D391A/N396A-FLAG from rice protoplasts completely lost its kinase activity (Fig. 4c).

Because OSK1 was phosphorylated by XopC2, the interaction between XopC2 and OSK1 was further explored. In pulldown assays, the lysates of E. coli cells co-expressing His6-OSK1 with GST-XopC2 or GST-XopC2D391A were incubated with GST-beads. His6-OSK1 was simultaneously detected with GST-XopC2 and GST-XopC2D391A but not with GST1, on GST-beads (Fig. 4d). Furthermore, co-expression of OSK1-NLuc and CLuc-XopC2 fusion proteins in N. benthamiana produced a strong luminescence signal, whereas little signal was detected when OSK1-NLuc was co-expressed with CLuc-AvrB2. As an additional negative control, the expression of CLuc-XopC2 and rice protein pKW1502-NLuc did not produce any signal (Fig. 4e). These results showed that XopC2 interacts with OSK1 in vivo and in vitro.

Ser53 phosphorylation in OSK1 by XopC2 enhances disease susceptibility in rice. To investigate whether OSK1 phosphorylation has any effect on the stability of OsJAZ proteins, the phosphomimic mutants OSK1T32D-FLAG, OSK1S53D-FLAG, OSK1S92D-FLAG, and OSK1T149D-FLAG were transiently co-expressed with OsJAZ9-HA in rice protoplasts. Only OSK1S53D-FLAG caused an obviously reduced OsJAZ9-HA accumulation compared with OSK1 co-expression (Fig. 5a), indicating that the S53D mutation in OSK1 promotes the degradation of JAZ proteins. Therefore, we developed an in vitro JAZ ubiquitination system40–42 to test whether OSK1 phosphorylation at Ser53 affects JAZ ubiquitination (Fig. 5b). First, Cullin1a was incubated with UBA3, AXR1, UBC12, RBX1, Rub1, and DCN1 for Cullin1a rubulation and activation. The Rub1-modified OsCullin1a was then incubated with OsUBA1, UBC5-HA, HA-ubiquitin, OsC011b, OsJAZ9, and different OSK1 variants in the ubiquitination buffer. Only OSK1S53D out of four phosphosite mutants had an enhanced ability to ubiquitylate OsJAZ9 compared with OSK1 (Fig. 5b). Moreover, we revealed that XopC2-HA, but not XopC2D391A-HA, co-expressed in rice protoplasts significantly promoted serine phosphorylation of OSK1T32A/S92A/T149A-FLAG. By contrast, OSK1T32A/S53A/S92A/T149A-FLAG phosphorylation was not altered by XopC2-HA or XopC2D391A-HA (Supplementary Fig. 9b). Next, we developed a polyclonal antibody that specifically detects Ser53 phosphorylation in OSK1. The in vitro kinase assays showed that XopC2, but not XopC2D391A, strongly phosphorylated OSK1 at Ser53 (Fig. 5c). Besides, induced expression of XopC2 caused OSK1 phosphorylation at Ser53 in the transgenic lines revealed by immunoblotting (Fig. 5d). The infection of the wild-type Xoc and C-ΔxopC2 strains induced obvious Ser53 phosphorylation in rice, whereas the ΔxopC2 and xopC2D391A-complemented strains did not (Fig. 5e). We further demonstrated that Ser53 phosphorylation of OSK1 in the OsJAZ9-HA-NE-2 transgenic line became to be detectable at 6 h after inoculation with the wild-type Xoc and C-ΔxopC2 strains, while no OSK1 phosphorylation occurred after ΔxopC2 and xopC2D391A inoculation (Supplementary Fig. 9c). These results demonstrated that Ser53 in OSK1 is a major phosphorylation site by XopC2.

To test whether Ser53 phosphorylation in OSK1 contributes to disease susceptibility in rice, we generated the transgenic rice plants expressing phosphomimic OSK1T32D driven by its native promoter and a maize ubiquitin promoter (Supplementary Fig. 9d). After spray inoculation with ΔxopC2, the OSK1T32D-expressing transgenic plants exhibited significantly greater bacterial populations than the wild-type and OSK1-expressing transgenic plants (Fig. 5f and Supplementary Fig. 9e). Besides,
expression of OSK1S53D, but not of OSK1, significantly compromised stomatal closure in transgenic plants after ΔxopC2 and Xoc infection and enhanced expression of JA-responsive genes after exogenous MeJA treatment (Fig. 5g-i and Supplementary Fig. 9f-h). The results showed that Ser53 in OSK1 is the preferred and biologically relevant phosphorylation site by XopC2 in vivo. Collectively, our findings indicate that XopC2 phosphorylates OSK1 at Ser53 to promote bacterial virulence.

The enhanced binding affinity of phosphomimic OSK1S53D to COI1b is dependent on the Arg13 residue in COI1b. OSK1 functions as a linker between Cullin1 and F-box proteins. Therefore, we investigated whether the S53D mutation affects the binding affinity of OSK1 to OsCullin1a and OsCOI1b. Co-immunoprecipitation (co-IP) assays showed that OsCOI1b-FLAG immunoprecipitated much more OSK1S53D-HA than OSK1-HA while similar amounts of OSK1S53D-HA and OSK1-HA were precipitated with OsCullin1a (Fig. 6a and Supplementary Fig. 10a). Besides, microscale thermophoresis (MST) assays showed that GST-OSK1S53D had a significantly higher binding affinity to His6-OsCOI1b (Kd = 12.705 ± 4.611 nM) than GST-OSK1 (Kd = 53.798 ± 12.263 nM) (Fig. 6b). The results indicate that the S53D mutation greatly enhances OSK1 binding to OsCOI1b, but does not alter its binding affinity to OsCullin1a.

To understand how Ser53 phosphorylation enhances the binding affinity of OSK1 to OsCOI1b, structure modeling was performed for OsCOI1b-OSK1-Cullin1-RXB1 and multiple F-box-OSK1 complexes (Supplementary Fig. 10b, c). Unlike F-box proteins OsTIR1 and D3, AtCOI1 and OsCOI1b carry the N-terminal flexible tails (Supplementary Fig. 10c). We speculate that the flexible tail of OsCOI1b containing positively charged
Arg9, Arg10, and Arg13 residues are sterically close to Ser53 in OSK1, and is involved in the interaction with pSer53 in OSK1 (Fig. 6c). To test this hypothesis, in vitro GST pulldown assays were performed using His6-tagged OsCOI1bR9A, OsCOI1bR10A, and OsCOI1bR13A. Compared with OsCOI1b, OsCOI1bR9A, and OsCOI1bR10A that bound to OSK1SSS3 more tightly than to OSK1, OsCOI1bR13A did not exhibit an enhanced affinity to OSK1SSS3 (Fig. 6d), indicating that Arg13 is essential for the enhanced binding of OsCOI1b to OSK1SSS3. These results showed that Ser53 phosphorylation enhances the binding affinity of OSK1 to COI1b and promotes the formation of SCF-OsCOI1b complex, which depends on Arg13 in OsCOI1b.

**Discussion**

Phytopathogenic bacteria utilize effectors to promote infection by disarming plant immunity and regulating plant cellular processes. In this study, we provide evidence that XopC2 is a type of T3S effector kinase. XopC2 specifically phosphorylates a highly conserved adaptor protein OSK1 in SCF complexes. The phosphorylation of OSK1 at Ser53 enhances its binding affinity to OsCOI1b, which targets OsJAZ for ubiquitination and proteasome-mediated degradation. As a consequence, JA signaling is promoted to suppress stomatal closure, which facilitates Xoc infection.

Multiple canonical and non-canonical bacterial effector kinases have been identified to phosphorylate host target proteins and inhibit host immunity. Despite no apparent similarity to any identified effector kinases at the primary sequence level, XopC2 and its homologs were predicted to possess a putative catalytic motif and P-loop-like ATP-binding motif through PSI-BLAST and HHpred searches (Fig. 1a and Supplementary Fig. 1). The kinase activity of XopC2 was subsequently confirmed by in vitro kinase assays, which demonstrated that XopC2 has the ability to autophosphorylate and phosphorylate OSK1 (Fig. 1c and Fig. 4a, c). Besides the N-terminal unordered region, XopC2 possesses a much longer kinase domain than canonical kinases (Fig. 1b; Supplementary Fig. 1). Sequence alignment showed that the kinase domain of XopC2 greatly differs from those of previously identified bacterial effector kinases except the conserved catalytic triad (Fig. 1), indicating that XopC2 and its homologs represent a novel family of kinase proteins.

Recombinant XopC2 exhibited a much weaker kinase activity than immunoprecipitated XopC2 expressed in rice protoplasts (Fig. 4a, c), suggesting that full activity of XopC2 may require one or more host co-factors. Alternatively, in vitro purified XopC2 may not be correctly folded or properly modified. The requirement of host factors for maximal activity has been documented for other kinases or kinase-fold-like effector proteins. For example, HSP90 is essential for the kinase activity of HopBF1. Similarly, ubiquitin-binding stimulates the kinase activity of OspgG47. Recent studies showed that calmodulin is a vital co-factor for the glutamylase activity of SidJ, a kinase-fold-like effector protein. The nature of the putative co-factors will be one focus of further study.

Disabling host stomatal immunity is often a prerequisite for successful entry into leaf tissues by various phytopathogenic bacteria including *P. syringae* (49). The pathogen suppresses stomatal defense through activating JA signaling with the action of multiple virulence factors, including coronatine, HopZ1, and HopX1. X. oryzae pathogens do not produce coronatine, nor does it encode homologs of HopZ1 and HopX1. Our data clearly showed that XopC2 in *Xanthomonads* is functionally equivalent to these effectors in suppressing stomatal immunity.

The SCF ligase complexes, which are conserved functionally and structurally in mammals and in plants, are usually comprised of a ubiquitinin-conjugating enzyme E2, a RING finger protein Rbx1, a scaffold protein Cullin1, a crucial adaptor Skp1, and a variable F-box protein. In mammals, Skp1 links the core Cullin-RING skeleton to diverse F-box proteins and thus forming different SCF E3 ligase complexes to mediate diverse protein degradation. Although 32 Skp1 homologs are encoded in rice, OSK1 shares the highest sequence similarity with mammal Skp1 and functions as a major adaptor protein in multiple hormone signaling pathways. Here, we revealed that OSK1 is the only protein in the SCF-OsCOI1 complex that was specifically phosphorylated by XopC2 (Fig. 4a, c; Supplementary Fig. 8a). The physical interaction of XopC2 and OSK1 was subsequently confirmed by pulldown and luciferase complementation imaging assays (Fig. 4d, e). However, we failed to detect the interaction by co-immunoprecipitation probably because of the transient nature of kinase-substrate interactions, which often prevents the identification of kinase substrates in co-IP assays. Although 32 Skp1 homologs are encoded in rice, OSK1 shares the highest sequence similarity with mammal Skp1 and functions as a major adaptor protein in multiple hormone signaling pathways. Here, we revealed that OSK1 is the only protein in the SCF-OsCOI1 complex that was specifically phosphorylated by XopC2 (Fig. 4a, c; Supplementary Fig. 8a). The physical interaction of XopC2 and OSK1 was subsequently confirmed by pulldown and luciferase complementation imaging assays (Fig. 4d, e). However, we failed to detect the interaction by co-immunoprecipitation probably because of the transient nature of kinase-substrate interactions, which often prevents the identification of kinase substrates in co-IP assays. Interestingly, expression of the JA-responsive genes, but not auxin-responsive and strigolactone-responsive genes, was enhanced in the XopC2-expressing transgenic seedlings (Fig. 3a, b, and Supplementary Fig. 5h, i), indicating that XopC2-mediated OSK1 phosphorylation specifically promotes JA signaling.
Furthermore, our data showed that much more OSK1 in rice was phosphorylated after _Xoc_ infection compared with _ΔxopC2_ infection (Fig. 4b), indicating that _Xoc_-secreted XopC2 enhances OSK1 phosphorylation during infection. Subsequently, four phosphosites were identified in OSK1 by in vitro kinase assays (Supplementary Fig. 8d). Among four phosphomimic OSK1 variants, only OSK1 S53D promoted SCF OsCOI1b-mediated OsJAZ9 ubiquitination and degradation (Fig. 5a, b). Ser53 phosphorylation in OSK1 catalyzed by XopC2 was confirmed via in vitro kinase assays and was detected in vivo by immunoblotting with a specific anti-pSer53 antibody (Fig. 5c–e). Time course assays showed that XopC2-mediated Ser53 phosphorylation in OSK1 occurred at the very early infection stage (Supplementary Fig. 9c), which is consistent with this finding that OsJAZ9 degradation became evident at 6 h post- _Xoc_ inoculation (Fig. 3d; Supplementary Fig. 6b). These results indicate that XopC2-mediated Ser53 phosphorylation in OSK1 is involved in JAZ degradation and subsequent suppression of stomatal immunity. More convincingly, the transgenic rice lines with OSK1 S53D expression exhibited an attenuated stomatal immunity and enhanced JA signaling and disease susceptibility (Fig. 5f–i; Supplementary Fig. 9f, h). We showed that the S53D mutation dramatically enhanced the binding affinity of OSK1 to OsCOI1b, but not to OsCullin1a (Fig. 6a–b; Supplementary Fig. 10a). The Ser53 residue is highly conserved in rice Skp1-like homologs and ASK1 in Arabidopsis. Based on structure modeling, we speculate that the flexible N-terminal tail of OsCOI1 with no predicted 3-D structure might be sterically close to the Ser53 residue of OSK1.
**Fig. 5 XopC2 phosphorylates OSK1 at Ser33 to enhance disease susceptibility in rice.** a OsJAZ9-HA accumulation was significantly reduced when co-expressing with OSK1S53D-FLAG compared with expressing alone or with FLAG-tagged OSK1, OSK1T32D, OSK1S92D, and OSK1T149D in rice protoplasts. The chart shows the OsJAZ9-HA levels normalized to β-Actin as actin. With OsJAZ9-HA, OSK1S53D, OSK1T32D, and OSK1S92D in rice protoplasts. The chart shows the OsJAZ9-HA levels normalized to β-Actin as actin. b OsJAZ9-HA was phosphorylated at Ser53 by XopC2 in vitro and in vivo. His6-XopC2 or His6-XopC2ΔXopC2 was incubated with His6-OSK1 for in vitro kinase assays (c). The IE transgenic lines were treated with DEX (30 μM) or buffer (mock) before protein extraction. d, e OSK1 was phosphorylated at Ser53 in rice after Xoc infection. The wild-type plants were inoculated as described in Fig. 4b. In e-a, Ser33 phosphorylation was detected by immunoblotting with an anti-pSer53 polyclonal antibody. f Bacterial population sizes in the ΔxopC2-inoculated leaves of the wild-type and OSK1-NE-1/12 and OSK1S53D-NE-1/6 transgenic seedlings expressing OSK1 and OSK1S53D driven by the native promoter. The wild-type and transgenic rice lines were spray-inoculated as described in Fig. 2a. g Stomatal conductance in the leaves of the wild-type, OSK1-NE and OSK1S53D-NE transgenic plants after ΔxopC2 challenging. The experiment was performed as described in Fig. 2b. h, i, MeA-induced expression of the JA-responsive gene OsLOX2 (h) and OsJAZ8 (i) in the wild-type, OSK1-NE, and OSK1S53D-NE transgenic plants. Data are shown as means ± SE (n = 3 independent experiments in a, b, n = 3, 8, 3 and 3 technical replicates per measurement in f, g, and i, respectively). All experiments were independently repeated 3 times with similar results in the panels a-i. The letters (a–b) in the panels a and f-i indicate statistically significant differences as revealed by one-way ANOVA, Tukey’s honest significant test.
SA and JA extraction and quantification. Endogenous JA and SA were extracted from 4-week-old rice leaves\textsuperscript{59}. Briefly, the leaves collected from 4-week-old seedlings (120 mg) were ground in liquid nitrogen. The powder was incubated overnight with 1 ml of extraction buffer (90% methanol, 0.1% formic acid), which was dried by flowing nitrogen gas. The extracted compounds were dissolved in 100 µl of extraction buffer and were then diluted by 5 and 500 times for SA and JA measurement. The SA and JA contents were quantified using plant SA/JA ELISA Kits following the manufacturer’s instructions (ZK-8280 for SA and ZK-8014 for JA, Zhen Ke Biological Technology Co., Ltd, Shanghai).

Chlorophyll content measurement. The leaves collected from 4-week-old seedlings (100 mg) were ground in liquid nitrogen to powder, which was incubated with 1 ml of acetone for 30 min in the dark. After centrifugation at 13, 400 × g for 10 min, 400 µl of the supernatant was diluted in 3.2 ml acetone. The chlorophyll content was measured by a spectrophotometer and was calculated using the equation, chlorophyll (mg l\(^{-1}\)) = 20.2 OD\(_{645}\) + 8.02 OD\(_{663}\)\textsuperscript{60}.

Virulence assays. Virulence of Xoc strains to rice was determined by spray and pressure inoculation\textsuperscript{61,62}. For pressure inoculation, the wild-type Xoc RS105 and ΔxopC2 strains were re-suspended with 10 mM MgCl\(_2\) to an OD\(_{600}\) of 0.3. Cell suspensions were pressure-inoculated into rice leaves using needleless syringes. The lesion length, the average of at least ten inoculated leaves, was measured at 14 days after Xoc inoculation. For spray inoculation, 3-week-old rice seedlings were pretreated with mock or 30 μM DEX supplemented with 0.01% Silwet L77 at 24 h before inoculation. Xoc strains were adjusted to an OD\(_{600}\) of 0.8 in 10 mM MgCl\(_2\) with 0.01% Silwet L77 and were then sprayed onto rice seedlings. The seedlings were kept in a high humidity chamber (over 90%). After 4 days post-inoculation (dpi), 3 pieces of 5 cm-long leaves with 3 technical repeats were detached from the inoculated seedlings and ground in sterile water after surface sterilization with 75% ethanol. The samples were spread on NA plates after serial dilution. Colony-forming units were counted after 2-day culturing.

**Fig. 6** The enhanced binding affinity of phosphomimic OSK\textsuperscript{S53D} to COI1b is dependent on the Arg\textsuperscript{13} residue in COI1b. 

a. OSK\textsuperscript{S53D}-HA bound to OsCOI1b-FLAG much stronger than OSK1-HA in co-IP assays. OSK1-HA and OSK1\textsuperscript{S53D}-HA were individually co-expressed with OsCOI1b-FLAG in rice protoplasts. The immunocomplex and input proteins were analyzed by immunoblotting using anti-HA and anti-FLAG antibodies. The experiments were independently repeated 3 times with similar results. 
b. His\textsubscript{6}-OSK1\textsuperscript{S53D} bound to OsCOI1b much stronger than His\textsubscript{6}-OSK1 in MST assays. OsCOI1b bound to His\textsubscript{6}-OSK1\textsuperscript{S53D} with a K\textsubscript{d} of 12.705 ± 4.611 nM while to GST-OSK1 with a K\textsubscript{d} of 53.798 ± 12.263 nM. Data are shown as means ± SE (n = 3 technical replicates). 
c. The 3-D structure of OsCOI1b-OSK1 was constructed via homology modeling with the ASK1-COI1-JAZ complex (PDB: 3OGM) as a template. The N-terminal flexible tail of OsCOI1b missing from the template was arbitrarily labeled. The N-terminal tail containing multiple positive-charged Arg residues is predicted to be adjacent to Ser\textsuperscript{53} in OSK1. OsCOI1bR\textsuperscript{13A} had a similar binding affinity to OSK1 and OSK1\textsuperscript{S53D}. In vitro-purified His\textsubscript{6}-TF-SUMO-tagged OsCOI1b\textsuperscript{R13A}, OsCOI1b\textsuperscript{R10A} and OsCOI1b\textsuperscript{R9A} were individually incubated with GST-OSK1 and GST-OSK1\textsuperscript{S53D}, respectively. After GST pulldown assays, the input and pulldown were detected with anti-His and anti-GST antibodies. The experiments were independently repeated 3 times with similar results.

SA and JA extraction and quantification. Endogenous JA and SA were extracted from 4-week-old rice leaves\textsuperscript{59}. Briefly, the leaves collected from 4-week-old seedlings (120 mg) were ground to powder in liquid nitrogen. The powder was incubated overnight with 1 ml of extraction buffer (90% methanol, 0.1% formic acid), which was dried by flowing nitrogen gas. The extracted compounds were dissolved in 100 µl of extraction buffer and were then diluted by 5 and 500 times for SA and JA measurement. The SA and JA contents were quantified using plant SA/JA ELISA Kits following the manufacturer’s instructions (ZK-8280 for SA and ZK-8014 for JA, Zhen Ke Biological Technology Co., Ltd, Shanghai).
Fig. 7 A working model for XopC2 function in promoting JA signaling and suppressing stomatal immunity. During Xoc infection, XopC2 is secreted into host cells. As a protein kinase, XopC2 specifically phosphorylates the Ser53 residue in OSK1, an essential component of SCF<sub>OsCOI1b</sub> complex. This enhances ubiquitination and degradation of JAZ proteins and promotes JA signaling and stomatal reopening. Therefore, stomatal defense is attenuated for the successful entry of the pathogen.

Stomatal conductance measurement. Three-week-old xopC2 IE transgenic seedlings were pretreated with 30 μM DEX or mock at 24 h before spray inoculation of Xoc strains. At 2 dpi, stomatal conductance was measured using a photosynthesis system (Model LI-6400XT, Li-Cor Inc., Lincoln, NE, USA)18. Briefly, stomatal conductance was measured with a 2 x 3 cm leaf chamber supplied with a red-blue LED light source, and parameters were set as 400 μmol mol<sup>−1</sup> CO<sub>2</sub> and 200 μmol m<sup>−2</sup> s<sup>−1</sup> photosynthetic photon flux density (PPFD).

Protein degradation assays in rice protoplasts. The ORFs of OsJAZ genes were amplified with the primers listed in Supplementary Table 1 and sub-cloned into pUC19-35S::3HA. The constructed plasmid was confirmed by sequencing. After being isolated using the GoldHi EndoFree Plasmid Maxi Kit (CWBIO), the plasmid DNA was incubated with 200 μg plasmid DNA per ml of PEG4000/CaCl<sub>2</sub> for 10 min. The transfected protoplasts were incubated in the W5 buffer (154 mM NaCl, 125 mM CaCl<sub>2</sub>, 5 mM KCl and 2 mM MES) supplemented with or without 30 μM DEX and 25 μM MG132 overnight. XopC2-FLAG and OsJAZs-HA were detected by immunoblotting with anti-FLAG (Sigma-Aldrich, F1804, 1:5,000) and HRP-conjugated anti-HA monoclonal antibodies (Roche, 11667475001, 1:2,000), respectively. The detection of OsActin using anti-Actin monoclonal antibody (Sigma-Aldrich, A2066, 1:5,000) was used as a loading control.

The ORF of OSK1 was amplified with OSK1-FLAG-Kpn I-F and OSK1-FLAG-Xba I-R (Supplementary Table 1) and was then sub-cloned into pUC19 with the 35S promoter after digestion by Kpn I and Xba I. The OSK1 mutant vectors were generated based on pUC19-35S:OSK1-FLAG via site-directed mutagenesis44. All constructs were confirmed by sequencing. The pUC19-35S:OsJAZ9-3HA plasmid was co-transfected with pUC19-35S::OSK1-FLAG, its phosphomimic variants, and pUC19 individually. The transfected cells were incubated in the W5 buffer for 10 min and were then broken in the lysis buffer containing 154 mM NaCl, 125 mM CaCl<sub>2</sub>, 5 mM KCl and 2 mM MES supplemented with 50 μM DEX and 25 μM MG132 overnight. XopC2 and OsJAZ9-HA were detected by immunoblotting with anti-HA monoclonal antibodies (Roche, 11667475001, 1:2,000), respectively. The detection of OsActin using anti-Actin monoclonal antibody (Sigma-Aldrich, A2066, 1:5,000) was used as a loading control.

OsJAZ9-HA degradation assay in transgenic rice plants during Xoc infection. Three-week-old transgenic rice seedlings expressing OsJAZ9-HA were challenged with Xoc strains by spray inoculation. Three 2 cm-length leaf pieces were collected at indicated timepoints after inoculation and ground into powder in liquid nitrogen. Total proteins were extracted with 400 μl of SDS sample loading buffer. OsJAZ9-HA was detected by immunoblotting with HRP-conjugated anti-HA monoclonal antibodies (Roche, 11667475001, 1:2,000).

Protein expression in E. coli and purification. The ORFs of xopC2, OSK1, OsRBR1, OsUBA3, OsAXR1, OsUBC12, OsRXB1, OsUBA1, and OsUBA3 were amplified with the primers (Supplementary Table 1) and were then sub-cloned into pET28a. OsJAZ9 and OsJAZ9-FLAG were cloned into pET32b. OsJAZ9, Upl1, and XopC2 were cloned into pGEX-4T-3. OsUBA1 was cloned into pCold-Sumo. OsCOI1b and OsCullin1a were cloned into pCold-TF. SUMO-OsCullin1a and SUMO-OsCOI1b fragments were generated using fusion PCR and sub-cloned into pCold-TF carrying His6 tag to generate pCold-TF-SUMO-OsCullin1a and pCold-TF-SUMO-OsCOI1b. The vectors carrying xopC2, OSK1 and OsCOI1b mutants were generated via site-directed mutagenesis. All constructs confirmed by sequencing were transformed into E. coli BL21(DE3) for protein expression.

For in vitro protein purification, 100 μg of IPTG was added into cell cultures to induce protein expression at a cell density of OD<sub>600</sub> = 0.6. The cells were further cultured at 16 °C with shaking at 150 rpm overnight. Cell cultures were collected by centrifugation at 1, 300 × g for 10 min and were then broken in the lysis buffer containing 50 mM Tris-Cl, pH 8.0, 150 mM NaCl, and 10 mM imidazole for His-tagged proteins or in the binding buffer containing 50 mM Tris-Cl, pH 8.0, and 150 mM NaCl for GST-tagged proteins. Cell debris was removed by centrifugation at 13, 400 × g for 10 min at 4 °C, and the supernatants were loaded onto Ni-NTA His·Bind<sup>®</sup> resin (Navogen, EMD Millipore, Billerica, MA), respectively. For His-tagged proteins, the resin was washed twice with lysis buffer and twice with washing buffer (50 mM Tris-Cl, pH 7.5, 150 mM NaCl, and 20 mM imidazole), and then was eluted with 2 ml of elution buffer (50 mM Tris-Cl, pH 7.5, 150 mM NaCl, and 250 mM imidazole). For GST-tagged proteins, the resin was washed four times.
In vitro kinase assay. In vitro kinase assays were performed as previously described with some modifications9. His6-XopC2 and variant proteins (5 μg) were incubated at 28 °C for 1 h in the reaction buffer containing 50 mM Tris-Cl, pH 7.5, 10 mM MgCl₂, protease inhibitor cocktail, 100 μM MG132, and 50 mM COR. After incubation, the ubiquitination was detected by immunoblotting with HRP-conjugated anti-HA (Roche, 11667475001, 1:2,000) and HRP-conjugated anti-His antibodies (CWBio, CW0285, 1:5,000).

In vitro JA29 ubiquitination assay. SCF(CUC1b) mediated JA29 ubiquitination was performed in vitro66. His6-TF-SUMO-OsCullin1a (1 μM) was incubated with His6-OsJAZ9-FLAG, 1 μM His6-OsJAZ9R1, 1 μM His6-OsJAZ9Ub1, and 1 μM GST-UPl1 at 28 °C for 1 h in a 400 μl reaction solution containing 50 mM Tris-Cl, pH 7.5, 5 mM ATP, 5 mM MgCl₂, 50 mM corona, and 0.5 mM DTT. The reaction mixture was equally divided into centrifuge tubes and was further incubated with different combinations of 50 nM His6-SUMO-UbA1A, 100 nM UBCH5a, 2 μM HA ubiquitin, 1 μM His-OsJAZ9-FLAG, 1 μM His6-TF-SUMO-OsCullin1b, and 1 μM His6-OsK1 or His6-OsK1 variants for 90 min. His6-OsJAZ9-FLAG ubiquitination was detected by immunoblotting with an anti-FLAG monoclonal antibody (Sigma-Aldrich, F1804, 1:2,000).

Identification of phosphosites in OsK1 via LC-MS/MS. The phosphosites in OsK1 were determined as described previously65. Briefly, in vitro purified His6-OsK1 (50 μg) and His6-XopC2 (25 μg) were incubated together in the buffer containing 50 mM Tris-Cl, pH 7.5, 5 mM ATP, 5 mM MgCl₂ and 1 mM DTT for 4 h at 28 °C. Proteins were digested with trypsin over night at 37 °C and separated by a Waters nanoHPLC nanoESI-RPS (Waters, Milford, MA). The nanoESI-MS/MS analysis was performed with a Thermo Q-Exactive high-resolution mass spectrometer (Thermo Scientific, Waltham, MA). The phosphorylated sites were analyzed by MS/MS spectra data with Mascot Distiller (Matrix Science, version 2.4).

Luciferase complementation imaging assay. Luciferase complementation imaging assay was performed through Agrobacterium-mediated transient expression in N. benthamiana leaves. The recoveries of HPLC (Waters, Milford, MA) and nanoESI-MS/MS were analyzed by LC/MS/MS spectrometry (Thermo Scientific, Waltham, MA).

In vitro JAZ9 ubiquitination assay. SCF(CUC1b) mediated JAZ9 ubiquitination was performed in vitro66. His6-TF-SUMO-OsCullin1a (1 μM) was incubated with washed 4 times with 1× PBS buffer. The ubiquitination was performed at 28 °C for 90 min in 40 μl reaction volume containing 10 μl of SCF(CUC1b)-bound beads, 100 ng E1, 300 ng E2, 2 μg HA-UB−/−, 1 μg His6-OsJAZ9−/−, 1 μg GST-XopC2−/− or GST-XopC2 mutant (as indicated in the buffer (50 mM Tris-Cl, pH 7.5, 2 mM ATP, 5 mM MgCl₂, 0.5 mM DTT, protease inhibitor cocktail, 100 μg MG132, and 50 mM COR). After incubation, the ubiquitination was detected by immunoblotting with HRP-conjugated anti-HA (Roche, 11667475001, 1:2,000) and HRP-conjugated anti-His antibodies (CWBio, CW0285, 1:5,000).
Hisa6-OSK1S53D in 1× PBS buffer supplied with 0.05% Tween 20. After incubation at 25 °C for 10 m, the mixtures were loaded onto standard-treated silica capillaries (NanoTip). Fluorescence was measured on 20% LED power and 20% IR laser power.

qRT-PCR analysis. Six-day-old wild-type and xopC2 transgenic rice seedlings were sprayed with 30 μM DEX for 4 h followed by treatment with 50 μM MeJA or mock control for 6 h. The seedlings were collected for RNA isolation using an ultrapure RNA extraction kit according to the manufacturer’s protocol (CWBio). Complementary DNA (cDNA) was synthesized by a reverse transcription system (Takara, Dalian, China) using total RNA as a template. Quantitative RT-PCR (qRT-PCR) was performed using an ABI PRISM® 7500 Sequence Detection System (Applied Biosystems, Carlsbad, CA, USA). The gene expression levels were calculated based on three repeats and were normalized against the expression of OsActin1 (Os03g0718100). The primers used for qRT-PCR were listed in Supplementary Table 1.

Statistics and reproducibility. The data in each figure are from representative experiments that were independently repeated at least three times. Statistical analyses were performed with a two-sided t-test, one-way ANOVA, following multiple comparisons of means with Tukey’s honest significance test or Duncan’s multiple range test.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability. All data and materials generated in the article are available from the corresponding author as request. Source data for figures and supplementary figures are provided in a Source data file. Source data are provided with this paper.

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References

1. Boller, T. & He, S. Y. Innate immunity in plants: an arms race between pattern recognition receptors in plants and effectors in microbial pathogens. Science 329, 742–744 (2009).
2. Deslandes, L. & Rivas, S. Catch me if you can: bacterial effectors and plant targets. Trends Plant Sci. 17, 644–655 (2012).
3. Galán, J. E. & Collmer, A. Type III secretion machines: bacterial devices for protein delivery into host cells. Science 284, 1322–1328 (1999).
4. Soucy, S. M., Huang, J. & Gogarten, J. P. Horizontal gene transfer: building the Xanthomonas world. F. A. Varden, F. A., del Conception, J. C., Maidment, J. H. & Bangert, M. J. Legionella. P. F. Lopez, V. A. et al. A bacterial effector mimics a host HSP90 client to targets. Proc. Natl Acad. Sci. 103, 5393–5398 (2006).
5. Horison, A., Chosed, R., Shu, H., Orth, K. & Mudgett, M. B. Xanthomonas type III effector XopD targets SUMO-conjugated proteins in planta. Microbiol. 50, 377–389 (2003).
6. Medina, C. A. et al. The role of type III effectors from Xanthomonas axonopodis pv. manihotis in virulence and suppression of plant immunity. Mol. Plant Pathol. 19, 593–606 (2018).
7. Teper, D. et al. The type III effector of XopX harbours E3 ubiquitin-ligase activity that is required for virulence. New Phyto. 220, 219–231 (2018).
8. Singer, A. U. et al. A pathogenic type III effector with a novel E3 ubiquitin ligase architecture. PLoS Pathog. 9, e1003213 (2013).
9. Schuebel, F. et al. 3′-NADP and 3′-NADAP, two metabolites formed by the bacterial type III effector AvrRox1. J. Biol. Chem. 291, 22868–22880 (2016).
10. Shidore, T. et al. The effect of AvrRox1 phosphorylates NAD in planta. PLoS Pathog. 13, e1006442 (2017).
11. Nino Liu, D. O., Rondel, P. C. & Bogdanove, A. J. Xanthomonas oryzae pv. oryzae pathogens: model pathogens of a model crop. Mol. Plant Pathol. 7, 303–324 (2006).
12. Melotto, M., Zhang, L., Oblessuc, P. R. & He, S. Y. Stomatal defense a decade later. Plant Physiol. 174, 561–571 (2017).
13. Gimenez-Ibanez, S. et al. The bacterial effector HopX1 targets JAZ transcriptional repressors to activate jasmonate signaling and promote infection in Arabidopsis. PLoS Biol. 12, e1001792 (2014).
14. Jiang, S. et al. Bacterial effector activates jasmonate signaling by directly targeting JAZ transcriptional repressors. PLoS Pathog. 9, e1003715 (2013).
15. Li, S. et al. The type III effector AvrB2 in Xanthomonas oryzae pv. oryzae suppresses rice immunity and promotes disease development. Mol. Plant Microbe Interact. 28, 869–880 (2015).
16. Cui, Y. et al. HrpE3 is a type III effector protein required for full virulence of Xanthomonas oryzae pv. oryzae in rice. Mol. Plant Pathol. 14, 678–692 (2013).
17. Xue, X., Zou, L., Liu, Z. & Chen, G. Identification of 17 Hrp-recognized proteins including multiple novel type III effectors. XOC, 3956 and XOC, 1550, in Xanthomonas oryzae pv. oryzae. PLoS ONE 9, e93205 (2014).
18. Kannan, N., Taylor, S. S., Zhao, Y., Venter, J. C. & Manning, G. Structural and functional diversity of the microbial kinome. PLoS Biol. 5, e17 (2007).
19. Howe, G. A., Major, J. T. & Koo, A. J. Modularity in jasmonate signaling for multistress resistance. Annu. Rev. Plant Biol. 69, 367–415 (2018).
20. Thines, B. et al. JAZ repressor proteins are targets of the SCG5 complex during jasmonate signalling. Nature 448, 661–665 (2007).
21. Schwechheimer, C. NEDD8 − its role in the regulation of Cullin-Ring ligases.Curr. Opin. Plant Biol. 45, 112−119 (2018).
22. Schwechheimer, C. & Merger, J. The NEDD8 modification pathway in plants. Front. Plant Sci. 5, 103 (2014).
23. Xin, X. F., Kvitko, B. & He, S. Y. Pseudomonas syringae what it takes to be a pathogen. Nat. Rev. Microbiol. 16, 316−328 (2018).
24. Kim, D. W. et al. The Shigella flexneri effector OspG interferes with innate immune responses by targeting ubiquitin-conjugating enzymes. Nat. Rev. Microbiol. 10, 14046–14051 (2005).
25. Navarro, L. et al. Identification of a molecular target for the Yersinia protein kinase A. Mol. Cell 26, 465−477 (2007).
26. Pham, T. H., Gao, X., Singh, G. & Hardwidge, P. R. Escherichia coli virulence protein NleH1 interaction with the v-Crk sarcoma virus CT10 oncogene-like protein (CRKL) governs NleH1 inhibition of the ribosomal protein S3 (RPS3)/ nuclear factor κB (NF-κB) pathway. J. Biol. Chem. 288, 34567−34574 (2013).
27. Zhou, Y., Dong, N., Hu, L. & Shao, F. The Shigella type three secretion system effector OspG directly and specifically binds to host ubiquitin for activation. PLoS ONE 8, e75598 (2013).
28. Gan, N. et al. Regulation of phosphoribosyl ubiquitination by a calmodulin-dependent glutamylase. Nature 527, 387−391 (2019).
29. Lotto, M., Underwood, W., Kocjan, J., Nomura, K. & He, S. Y. Plant stomata function in innate immunity against bacterial invasion. Cell 126, 969−980 (2006).
50. Nishiyama, K. & Ezuka, A. Species of bacteria producing coronatine, a new physiologically active substance. Annu. Phytopathol. Soc. Jpn. 44, 179–183 (1978).

51. Cardozo, T. & Pagano, M. The SCF ubiquitin ligase: insights into a molecular machine. Nat. Rev. Mol. Cell Biol. 5, 739–751 (2004).

52. Vierstra, R. D. The ubiquitin-26S proteasome system at the nexus of plant biology. Nat. Rev. Mol. Cell Biol. 10, 383–397 (2009).

53. Kong, H. et al. Patterns of gene duplication in the plant SKP1 gene family in angiosperms: evidence for multiple mechanisms of rapid gene birth. Plant J. 50, 873–885 (2007).

54. Kahloul, S. et al. Structural, expression and interaction analysis of rice SKP1-like genes. DNA Res. 20, 67–78 (2013).

55. de Oliveira, P. S. L. et al. Revisiting protein kinase-substrate interactions: toward therapeutic development. Sci. Signal. 9, e3 (2016).

56. Zhang, D., Tian, C., Yin, K., Wang, W. & Qiu, J. Postinvasive bacterial resistance conferred by open stomata in rice. Mol. Plant Microbe Interact. 25, 255–266 (2012).

57. He, F., Chen, S., Ning, Y. & Wang, G. L. Rice (Oryza sativa) protoplast isolation and its application for transient expression analysis. Curr. Protoc. Plant Biol. 1, 373–383 (2016).

58. Wang, S. et al. Rice OsFLS2-mediated perception of bacterial flagellins is evaded by Xanthomonas oryzae pv. oryzae and oryzyola. Mol. Plant 8, 1024–1037 (2015).

59. Wang, J. et al. The kinase OsCPK4 regulates a buffering mechanism that fines tunes innate immunity. Plant Physiol. 176, 1835–1849 (2018).

60. Okhi, Y., Funatsu, N., Konishi, N. & Chiba, T. The mechanism of poly-NEDD8 chain formation in vitro. Biochem. Biophys. Res. Commun. 381, 443–447 (2009).

61. Tan, P. et al. Recruitment of a ROC1-CUL1 ubiquitin ligase by Skp1 and HOS to catalyze the ubiquitination of Hrs. Mol. Cell 3, 527–533 (1999).

62. Chen, H. et al. Firefly luciferase complementation imaging assay for protein-protein interactions in plants. Plant Physiol. 146, 368–376 (2008).

63. Sambrook, J. & Russell, D. W. Detection of protein-protein interactions using the GST fusion protein pulldown technique. CSH Protoc. 2006, pro3757 (2006).

64. Hanks, S. K. & Hunter, T. Protein kinases 6. The eukaryotic protein kinase superfamily: kinase (catalytic) domain structure and classification. FASEB J. 9, 576–596 (1995).