Arabidopsis HFR1 Is a Potential Nuclear Substrate Regulated by the Xanthomonas Type III Effector XopD<sub>\textit{Xcc}8004</sub>

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Abstract

XopD<sub>\textit{Xcc}8004</sub>, a type III effector of Xanthomonas campestris pv. campestris (Xcc) 8004, is considered a shorter version of the XopD, which lacks the N-terminal domain. To understand the functions of XopD<sub>\textit{Xcc}8004</sub> in planta, a transgenic approach combined with inducible promoter to analyze the effects of XopD<sub>\textit{Xcc}8004</sub> in Arabidopsis was done. Here, the expression of XopD<sub>\textit{Xcc}8004</sub>, in Arabidopsis elicited the accumulation of host defense-response genes. These molecular changes were dependent on salicylic acid and correlated with lesion-mimic phenotypes observed in XVE::XopD<sub>\textit{Xcc}8004</sub> transgenic plants. Moreover, XopD<sub>\textit{Xcc}8004</sub> was able to desumoylate HFR1, a basic helix-loop-helix transcription factor involved in photomorphogenesis, through SUMO protease activity. Interestingly, the hfr1-201 mutant increased the expression of host defense-response genes and displayed a resistance phenotype to Xcc8004. These data suggest that HFR1 is involved in plant innate immunity and is potentially regulated by XopD<sub>\textit{Xcc}8004</sub>.

Introduction

Post-translational modifications enable plants to rapidly modify the stability and activity of key factors that play fundamental roles in defense signaling during bacterial infections [1], [2]. Studies have shown that sumoylation, a reversible and dynamic process, appears to be essential for suppressing defense responses in non-infected plants [3]. The sumoylation machinery is composed of small ubiquitin-like modifier (SUMO)-specific E1 (SAE1/SAE2 heterodimer), E2 (SCE1), and E3 (SIZ1 and MMS21/HPY2) to make SUMO conjugates [4], [5]. Elevated accumulation of salicylic acid (SA) accompanied by localized programmed cell death in sum1sum2 and siz1mutants exhibits increased resistance to Pseudomonas syringae and suggests that
sumoylation machinery is likely to contribute to systemic-acquired resistance (SAR), resulting in enhanced resistance against further pathogen attacks [6–8].

The plant immune system is a multilayered type of immune response, which contains pathogen-associated molecular pattern-triggered immunity and effector-triggered immunity [9], [10]. To overcome the complex immune system, pathogens secrete or inject a range of effectors into host cells to manipulate host cellular functions and alter host defense responses [11], [12]. Although the functions of these virulence factors remain largely unknown, an increasing body of evidence demonstrates that pathogens employ a strategy to structurally or functionally mimic host cellular activities [13], [14]. In the past years, several bacterial effectors have been found to share structural similarity with SUMO proteases. Because bacteria do not have a SUMO system, it would be interesting to understand the role of pathogen effectors employing SUMO protease activity.

Previous studies have shown that the Xanthomonas type III effector XopD possesses desumoylation activity and localizes to nuclear foci in plant cells [15–17]. The subnuclear localization of XopD suggests that XopD may target SUMO-conjugated proteins in the plant nucleus. Indeed, XopD$_{Xcc100}$ from the strain B100 of Xcc specifically interacts with MYB30 to suppress its activity in activating plant defense responses required for anti-Xcc immunity [16]; XopD$_{Xcc85–10}$ from the strain 85–10 of Xanthomonas campestris pv. vesicatoria (Xcv) specifically interacts with SIERF4 to suppress its activity in activating ethylene-induced responses, which is required for anti-Xcv immunity [18]. XopD is composed of an N-terminal domain, ERF-associated amphiphilic repression motifs, and a C-terminal SUMO protease domain [17], [19]. Although the C-terminal domain of XopD has SUMO peptidase and isopeptidase activities, lacking the functional N-terminal domain fails to suppress MYB30-mediated defense responses or desumoylation of SIERF4 [16], [18]. Thus, the N-terminus of XopD is essential for the virulence of Xanthomonas in planta. However, in the Xcc8004 strain, XopD$_{Xcc8004}$ was considered as a XopD without N-terminal domain [17], [19]. Although XopD$_{Xcc8004}$ has been shown to be able to be secreted via a hrp-dependent pathway and contains functional SUMO protease activity, the action of XopD$_{Xcc8004}$ in planta is still largely unknown [19].

Recently, light has been considered as an important regulator in modulating plant immunity [20], [21]. The availability and quality of light affects the plant development, as well as influences the plant defense responses. For example, a high ratio of red to far-red light enhances plant resistance to herbivorous insects [22]; a low ratio of red to far-red light reduces plant resistance to bacterial pathogens [23], [24]. Thus, mutations in the photoreceptors greatly influence plant defense responses. In this study, an inducible expression system was used to study the functions of XopD$_{Xcc8004}$ in transgenic Arabidopsis plants. Finally, we showed that HFR1, a basic helix-loop-helix transcription factor involved in light-signaling pathway, is a potential nuclear substrate regulated by XopD$_{Xcc8004}$.

Materials and Methods

Plant materials and growth conditions

Arabidopsis thaliana was grown at 21°C under a 16-h light/8-h dark photoperiod for Agrobacterium transformations, and a 12-h light/12-h dark photoperiod for Xanthomonas spp. inoculations. N. benthamiana was grown at 26°C under a 16-h light/8-h dark photoperiod for Agrobacterium transient expression assay. The Arabidopsis WT, hfr1–201 mutant, and nahG transgenic plants are in the Columbia ecotype background [6], [25].

Plasmid constructions

Xcc8004 genomic DNA was used for amplification of the XC_1213 DNA fragment encoding XopD$_{Xcc8004}$. An A. thaliana cDNA library was used for the amplification of the At1g02340 DNA fragment.
DNA fragment encoding HFR1. DNA fragments amplified by PCR using AccuPrime pfx DNA polymerase (Invitrogen) were subcloned into appropriate vectors by restriction site reconstructions. For the generation of Arabidopsis transgenic plants, PCR products were subcloned into the pER8 vector under the control of the XVE promoter [26]. For subcellular localization assays, PCR products were subcloned into pBA-YFP or pBA-CFP vectors under the control of the Cauliflower mosaic virus 35S promoter [27]. For yeast two-hybrid assays, PCR products were subcloned into pGADT7 and pGBK7 vectors (Clontech) to generate AD-HFR1 and BK-XopD_{Xcc8004} constructs. For the expression of recombinant proteins, PCR products were subcloned into pET-SUMO (Invitrogen), pMAL-c2 (New England Biolabs), or pGEX4T-1 (GE Healthcare) vectors to produce N-terminal His-SUMO-, MBP-, or GST-tagged XopD_{Xcc8004}, HFR1, or AS2 proteins. The K72A mutant of HFR1 and the C355A mutant of XopD_{Xcc8004} were generated by QuikChange site-directed mutagenesis (Stratagene) according to the manufacturer’s instructions. For homologous recombination, the 985-bp upstream and 976-bp downstream regions of XopDXcc8004 were amplified from Xcc8004 genomic DNA and subcloned into a pK18mobsacB vector. To establish an in vitro sumoylation system, DNA fragments encoding Arabidopsis SAE1 (SAE1b), SAE2, and SCE1 were excised from the pACYCDuet-AtSAE1b-AtSAE2 and pCDFDuet-AtSUMO1(GG)-AtSCE1 plasmids [28], and subcloned into pET28a or pET29a vectors (Invitrogen) by restriction site reconstructions to produce His-tagged SAE1, SAE2, and SCE1 proteins. All plasmids were verified by DNA sequencing.

Arabidopsis transformations

To obtain Arabidopsis transgenic plants, plasmids were introduced into the Agrobacterium tumefaciens strain ABI by the freeze-thaw method [29] and then transformed into A. thaliana Col-0 using the floral-dip method [30]. Arabidopsis seeds were grown on half-strength Murashige and Skoog (1/2× MS) medium containing hygromycin (12.5 µg mL⁻¹) and carbenicillin (100 µg mL⁻¹) to obtain transgenic lines. Homozygous seeds were further selected and amplified for analyses.

Trypan blue staining

To characterize the lesion-mimic phenotype, Arabidopsis transgenic plants expressing XopD_{Xcc8004} were examined by trypan blue staining [31]. Briefly, transgenic seeds were germinated on 1/2× MS medium containing DMSO or 20 µM β-estradiol. Three-week-old seedlings were stained by boiling in a solution containing 10 mL of lactic acid, 10 mL of glycerol, 10 g of phenol, and 10 mg of trypan blue (dissolved in 10 mL of distilled water), and further destained in 2.5 g mL⁻¹ chloral hydrate solution. Images were collected with a Leica ZM75 microscope.

qRT-PCR

To measure Arabidopsis gene expression levels, total RNA was extracted by using the Trizol reagent and reverse transcribed into cDNAs using a Superscript III first-strand synthesis supermix according to the manufacturer’s instructions (Invitrogen). The qRT-PCR reactions were performed on an Eco real-time PCR system (Illumina) with the KAPA SYBR fast qPCR kit (Kapa Biosystems). Relative amounts of transcripts were normalized to the transcript level of a house keeping gene, EF1α. Experiments were repeated at least 3 times.

Recombinant protein purifications and antibody production

To produce recombinant proteins, all constructs were transformed into Escherichia coli BL21 (DE3) cells and cultured at 24°C until the optical density at 600 nm reached 0.4. Then,
isopropyl β-D-1-thiogalactopyranoside was added to a final concentration of 0.2 mM and cells were further incubated overnight. After cell lysis, bacterial cell extracts were purified using appropriate resins according to the manufacturer’s instruction. For in vitro sumoylation assays, Arabidopsis SAE1, SAE2, SCE1, and AtSUMO1 (with Gly-Gly at the C-terminus) proteins were purified using Ni²⁺-NTA resin (Qiagen). For in vitro pull-down assays, MBP- and GST-tagged proteins were purified using an amylose resin (New England Biolabs) and glutathione-Sepharose 4B (GE Healthcare), respectively. To generate a specific antibody against XopD<sub>Xcc8004</sub>, the His-SUMO-XopD<sub>Xcc8004</sub> protein was purified using a Ni²⁺-NTA resin and cleaved with Ubl-specific protease 1 (Ulp1) to remove the His-SUMO tag. After cleavage, proteins were purified by using a Sephacryl S-200 HR gel filtration column (GE Healthcare) to obtain the XopD<sub>Xcc8004</sub> protein alone. Finally, a rabbit polyclonal antibody raised against XopD<sub>Xcc8004</sub> was obtained by affinity purification using a polyvinylidene difluoride membrane as a coupling matrix [32].

Bacterial strains and inoculations

The Xcc8004 ΔXopD mutant strain was obtained using the sacB system [33]. Plasmid for homologous recombination was introduced into Xcc8004, and deletion mutant was verified by PCR. For bacterial inoculations, Xcc8004 spp. were cultured in nutrient broth supplemented with yeast extract (3 g of beef extract, 5 g of peptone, and 3 g of yeast extract in 1 liter of water) at 28°C. Four- to five-week-old Arabidopsis plants were used for bacterial growth assays. For XVE::XopDXcc8004 transgenic plants, leaves were infiltrated with a bacterial suspension (2 × 10<sup>6</sup> CFU mL<sup>–1</sup>) by using a syringe at 24 h after plants had been sprayed with 20 μM β-estradiol. After inoculation, plants were kept at 21°C in a growth chamber, and bacterial populations in leaves were determined at indicated time intervals using agar plates of nutrient broth supplemented with yeast extract containing rifampin (50 μg mL<sup>–1</sup>). Experiments were repeated at least 3 times.

Yeast two-hybrid assays

AD-HFR1 and BK-XopD<sub>Xcc8004</sub> constructs were transformed into the yeast strain AH109 by using the lithium acetate/single-stranded carrier DNA/polyethylene glycol method [34]. First, yeast cells were grown on synthetic-defined minimal yeast medium lacking leucine and tryptophan (Clontech) to maintain plasmids. Transformed colonies were further plated on synthetic-defined minimal yeast medium lacking leucine, tryptophan, and histidine (Clontech) to test the interaction between XopD<sub>Xcc8004</sub> and HFR1.

Subcellular localization assays

To examine the subcellular localization of HFR1-CFP and XopD<sub>Xcc8004</sub>-YFP, agroinfiltration was performed [35]. Briefly, A. tumefaciens stains carrying the 35S::HFR1-CFP or 35S::XopD<sub>Xcc8004</sub>-YFP plasmid were inoculated into N. benthamiana leaves. Fluorescence signals were observed by confocal laser scanning microscopy at 36 h after agroinfiltration, and images were collected with the Olympus Fluoview FV1000 system.

In vitro pull-down assays

GST pull-down experiments were performed by inoculating 2 μg of GST alone or GST-tagged proteins with 2 μg of MBP alone or MBP-tagged proteins in binding buffer (50 mM Tris-HCl at pH 7.5, 100 mM NaCl, 0.25% Triton X-100, 35 mM β-mercaptoethanol) for 2 h at room temperature. Next, 25 μL of glutathione-Sepharose 4B (GE Healthcare) were added, and
samples were further incubated for 1 h at room temperature. After extensive wash, pulled down proteins were eluted with 2.5× sample buffer and separated on a 10% sodium dodecyl sulfate-polyacrylamide gel. Western blotting was performed using an anti-MBP antibody to detect MBP-tagged proteins (Amersham). The chemiluminescence signals generated by the ECL reagent were further examined with the ImageQuant LAS4000 mini (GE Healthcare).

**In vitro sumoylation assays**

In *vitro* sumoylation was performed using purified recombinant proteins in a reaction buffer (50 mM Tris-HCl, pH7.4, 100 mM NaCl, 4 mM ATP, 10 mM MgCl₂, 4 mM DTT) in a total volume of 30 μL. After incubation for 2 h at 30°C, the reaction mixtures were separated on 10% sodium dodecyl sulfate-polyacrylamide gels. MBP-HFR1 and sumoylated MBP-HFR1 were detected by western blotting using an anti-MBP antibody. The chemiluminescence signals generated by the ECL reagent were further examined with the ImageQuant LAS4000 mini (GE Healthcare).

**RNA-Seq analysis**

To identify differentially expressed genes in XVE::XopD<sub>Xcc8004</sub> transgenic plants upon β-estradiol treatment, next-generation sequencing was done on the HiSeq 2000 (Illumina) using total RNA samples extracted with the RNeasy plant mini kit (Qiagen). For transcriptome analysis, sequence reads were aligned using CLC bio and gene expression levels were normalized as reads per kilobase of exon model per million mapped reads. Finally, the differentially expressed genes were identified by DEseq [36].

**Primers**

Primer sequences for plasmid constructions and qRT-PCR analyses are listed in S1 Table.

**Results**

Expression of XopD<sub>Xcc8004</sub> elicits a SA-mediated defense response in *Arabidopsis*

XopD<sub>Xcc8004</sub> from the Xcc strain 8004 is a shorter version of XopD, which lacks the N-terminal domain (Fig. 1A). To characterize the functions of XopD<sub>Xcc8004</sub> in plant cells, *Arabidopsis* transgenic plants carrying an inducible XVE::XopD<sub>Xcc8004</sub> transgene were generated. Here, a lesion-mimic phenotype associated with localized, necrotic spots was observed in XVE::XopD<sub>Xcc8004</sub> transgenic plants after β-estradiol (inducer) treatment (Fig. 1B, C). Further examination using trypan blue staining confirmed that lesion-mimics were formed because of cell death (Figs. 1B, S1), whereas transgenic plants harboring empty vector (XVE) did not show cell death phenotype upon β-estradiol treatment (S2 Fig.).

To examine whether XopD<sub>Xcc8004</sub> can trigger a SA-mediated defense response which may contribute to the hypersensitive cell death, genes involved in the SA-mediated defense-signaling network [37] were selected for quantitative reverse transcription-polymerase chain reaction (qRT-PCR) validation. Compared with dimethyl sulfoxide (DMSO) control treatment, genes involved in defense response (*AIG1* and *DOX1*), SA biosynthesis (*EDS5* and *SID2*), SA accumulation and downstream signaling (*WIN3*, *PAD4* and *WRKY70*), as well as SA-standard marker genes (*PR1*, *PR2* and *PR5*) were highly elevated in XVE::XopD<sub>Xcc8004</sub> transgenic plants after β-estradiol treatment (Figs. 1D, S1). By contrast, no significant difference was observed in XVE (vector only) transgenic plants (S2 Fig.). Next, we transfected XVE::XopD<sub>Xcc8004</sub> into an *Arabidopsis* transgenic line overexpressing bacterial salicylate hydroxylase (*nahG*). With the
expression of nahG, the cell death phenotype and SA response-related genes induced by XopDcc8004 were all inhibited by nahG (Figs. 1D, S3). These results suggest that XopDcc8004 elicits a SA-dependent defense response which may contribute to the lesion-mimic phenotype observed in XVE::XopDcc8004 transgenic plants.

To investigate the genome-wide expression profile of mRNAs in response to the expression of XopDcc8004 in Arabidopsis, the total RNA from XVE::XopDcc8004 transgenic seedlings upon DMSO and β-estradiol treatments were extracted for a comparative transcriptome
analysis. In summary, a total of 23.1 million reads mapped to the *Arabidopsis* genome were generated after quality trim and a total of 103 differentially expressed genes with \( p < 0.001 \) were identified using the DESeq method (S2 and S3 Tables). Among them, 85 genes were upregulated and 18 genes were downregulated after \( \beta \)-estradiol treatment. Functional annotations on the 85 up-regulated genes revealed that a total of 25 genes associated with defense responses including the SA-mediated response were highly induced by XopD \( Xcc8004 \) (Table 1).

**Suppression of Xcc8004 growth by XopD \( Xcc8004 \)**

Because the lesion-mimic phenotype accompanied by the up-regulation of SA response-related genes in \( XopD_{Xcc8004} \) transgenic plants resembles the hypersensitive response (HR) in pathogen infection, we hypothesize that the expression of \( XopD_{Xcc8004} \) in *Arabidopsis* may result in enhanced resistance against bacterial pathogens. In order to examine the effect of \( XopD_{Xcc8004} \) on the resistance of *Arabidopsis*, multiplication of the \( Xcc8004 \) strain on \( XVE::XopD_{Xcc8004} \) transgenic plants was tested after \( \beta \)-estradiol treatment. Compared with \( XVE \) transgenic plants, multiplication of the \( Xcc8004 \) strain was suppressed by the expression of \( XopD_{Xcc8004} \) in planta at 5 days post inoculation (dpi) (Fig. 2A).

### Table 1. Annotation of the differentially expressed genes (\( p < 0.001 \)) involved in plant defense responses.

| Name | AGI number | Base mean | log2 Fold Change | Functional annotations |
|------|------------|-----------|------------------|------------------------|
|      |            | \( \beta \)-estradiol | DMSO |                          |
| SBT3.3 | At1g32960 | 2.19     | 434.53 | 7.63 | Subtilase family protein |
| DIR5  | At1g64160 | 2.19     | 354.19 | 7.34 | Disease resistance-responsive family protein |
| AIG1  | At1g33960 | 20.81    | 850.80 | 5.35 | AvrRPT2-induced gene 1 |
| PCC1  | At3g22231 | 14.24    | 544.07 | 5.26 | Pathogen and circadian controlled 1 |
| TPS4  | At1g61120 | 5.48     | 262.91 | 5.58 | Terpene synthase 4 |
| AT5g10760 | 25.20 | 670.96     | 4.74 | Eukaryotic aspartyl protease family protein |
| GSTF3 | At2g02930 | 16.43    | 406.23 | 4.63 | Glutathione S-transferase F3 |
| AT5g03350 | 39.44 | 746.73     | 4.24 | Legume lectin family protein |
| GSTU4 | At2g29460 | 23.00    | 461.91 | 4.33 | Glutathione S-transferase tau 4 |
| NIT2  | At3g44300 | 261.81   | 3868.75 | 3.89 | Nitrilase 2 |
| RLP23 | At2g32680 | 12.05    | 272.95 | 4.50 | Receptor-like protein 23 |
| ELI3  | At4g37990 | 5.48     | 175.27 | 5.00 | Elicitor-activated gene 3–2 |
| ANK   | At1g13609 | 4.38     | 157.93 | 5.17 | Defensin-like (DEFL) family protein |
| CRK7  | At4g23150 | 1.10     | 102.24 | 6.54 | Cysteine-rich receptor-like protein kinase 7 |
| PR2   | At3g57260 | 47.10    | 709.30 | 3.91 | Beta-1,3-glucanase 2 |
| FMO1  | At1g19250 | 18.62    | 340.50 | 4.19 | Flavin-dependent monooxygenase 1 |
| CRK4  | At3g45860 | 8.76     | 189.88 | 4.44 | Cysteine-rich receptor-like protein kinase 4 |
| WAKL10| At1g79680 | 8.76     | 179.84 | 4.36 | Wall-associated kinase-like 10 |
| PDF1.4| At1g19610 | 27.39    | 355.11 | 3.70 | Arabidopsis defensin-like protein |
| PNP-A | At2g18660 | 12.05    | 191.70 | 3.99 | Plant natriuretic peptide A |
| RLP38 | At3g23120 | 2.19     | 85.81  | 5.29 | Receptor-like protein 38 |
| AT5g24200 | 4.38 | 109.54     | 4.64 | Alpha/beta-hydrolases superfamily protein |
| AT5g04220 | 9.86 | 163.40     | 4.05 | Disease resistance protein (TIR-NBS-LRR) family |

AGI, *Arabidopsis* Genome Initiative; Base mean, the number of reads divided by the size factor (normalization constant) of sample; Fold change, \( \beta \)-estradiol base mean/DMSO base mean.

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We further generated a xopD null mutant by homologous recombination to validate the function of XopD <i>Xcc</i>8004 in suppressing the virulence of <i>Xcc</i>8004. Here, <i>Arabidopsis</i> WT leaves inoculated with <i>Xcc</i>8004 ΔxopD strain exhibited a higher titer of bacteria at 5 dpi than those inoculated with the <i>Xcc</i>8004 strain (Fig. 2B). This phenotype was able to be complemented when <i>Xcc</i>8004 ΔxopD strain was transformed with a broad host plasmid (pBBR1) expressing XopD <i>Xcc</i>8004 (Fig. 2B). Taken together, these results suggest that XopD <i>Xcc</i>8004 acts as a negative factor in suppressing the growth of <i>Xcc</i>8004.

**XopD <i>Xcc</i>8004-eliciting defense responses are mainly dependent on the SUMO protease activity**

To examine whether the SUMO protease activity of XopD <i>Xcc</i>8004 is required for eliciting the plant immunity, XVE::XopD <i>Xcc</i>8004(C355A) transgenic plants were generated, in which mutation of the conserved cysteine (C355A) in XopD <i>Xcc</i>8004 was not able to hydrolyse the SUMO substrates. Compared with XVE::XopD <i>Xcc</i>8004 transgenic plants, no hypersensitive cell death was observed in XVE::XopD <i>Xcc</i>8004(C355A) transgenic plants after β-estradiol treatment (Figs. 1B, 3A, S4). Further investigation of the gene expression involved in defense responses revealed that the C355A mutation largely suppressed the ability of XopD <i>Xcc</i>8004 in eliciting the
expression of SA response-related genes in Arabidopsis (Figs. 3B, S4). Next, we transformed Xcc8004ΔXopD strain with a broad host plasmid to express XopD Xcc8004(C355A). However, Arabidopsis WT leaves inoculated with Xcc8004ΔXopD/XopD Xcc8004(C355A) strain still exhibited a higher titer of bacteria at 5 dpi as well as those inoculated with Xcc8004ΔXopD strain (Fig. 2B). These results suggest that the SUMO protease activity of XopD Xcc8004 is required for eliciting a SA-mediated defense response in Arabidopsis and suppressing the virulence of Xcc8004.

XopD Xcc8004 interacts with Arabidopsis HFR1

Unexpectedly, a long hypocotyl phenotype associated with the lesion-mimic phenotype was observed with the expression of XopD Xcc8004 in Arabidopsis (Figs. 4A). Compared with XVE transgenic plants, the hypocotyl length of XVE::XopD Xcc8004 seedlings reached to 4.5 mm at 14 days after germination in medium containing β-estradiol (Figs. 4B, S5). We noted that the long hypocotyl phenotype elicited by the expression of XopD Xcc8004 similar to those observed in siz1–2 carrying a partially functional SIZ1phd [38]. SIZ1phd contains a C134Y mutation in the zinc finger motif required for the SUMO E3 ligase activity of SIZ1. Cheong et al. found that expression of SIZ1phd in the siz1–2 mutant created a light-dependent long hypocotyl phenotype. Therefore, we investigated whether light signaling components were potential substrates of XopD Xcc8004. Here, several key components involved in the light signaling pathway including HY5, LAF1, Fin219, PAT3, SPA1, and HFR1 were analyzed for their interaction with XopD Xcc8004 using the yeast two-hybrid assay. Among them, HFR1, a basic helix-loop-helix transcription factor, showed a positive interaction with XopD Xcc8004 in selection medium lacking tryptophan/leucine/histidine (Fig. 5A).

Next, we investigated whether XopD Xcc8004 can colocalize with HFR1 in plant cells. HFR1 has been shown to localize to nucleus in subnuclear foci [27], whereas XopD Xcc8004 was localized to the nucleus in a homogeneous pattern [17]. If XopD Xcc8004 can interact with HFR1, the

Figure 3. XopD Xcc8004(C355A) loses the activity for activating plant immunity. (a) Morphological examination and trypan blue staining of two-week-old leaves of Arabidopsis XVE::XopD Xcc8004(C355A) transgenic plants. Scale bar: 1 mm. (b) The expression levels of genes involved in the SA-mediated defense signaling network were examined by qRT-PCR and normalized to EF1α. The relative expression levels of each gene in the DMSO control were set at 1.
coexpression of HFR1 may cause the relocalization of XopD<sub>Xcc8004</sub> to HFR1-containing nuclear foci. Here, we coexpressed XopD<sub>Xcc8004</sub>-yellow fluorescence protein (YFP) and HFR1-cyan fluorescent protein (CFP) in <i>Nicotiana benthamiana</i> cells using agroinfiltration and found that XopD<sub>Xcc8004</sub>-YFP was colocalized with HFR1-CFP in nuclear foci (Fig. 5B). To further examine whether XopD<sub>Xcc8004</sub> can directly interact with HFR1 <i>in vitro</i>, a pull-down assay was performed with purified recombinant proteins. Fig. 5C shows that maltose-binding protein (MBP)-HFR1 was specifically pulled down by glutathione S-transferase (GST)-XopD<sub>Xcc8004</sub> but not by GST alone. By contrast, no signal was observed when the negative control proteins MBP and MBP-AS2 were used in the assay (Fig. 5C).

To examine whether the long hypocotyl phenotype observed in <i>XVE::XopDXcc8004</i> seedlings is dependent on the SUMO protease activity of XopD<sub>Xcc8004</sub>, we investigated the phenotype of <i>XVE::XopDXcc8004(C355A)</i> transgenic plants. However, a long hypocotyl phenotype was also observed in <i>XVE::XopDXcc8004(C355A)</i> seedlings (S5 Fig.). This result suggests that the long hypocotyl phenotype caused by XopD<sub>Xcc8004</sub> is not simply due to the SUMO protease activity.

**K72 in HFR1 is desumoylated by XopD<sub>Xcc8004</sub> <i>in vitro</i>**

Because XopD<sub>Xcc8004</sub> has been shown to possess SUMO protease activity [16], [17], the interaction between XopD<sub>Xcc8004</sub> and HFR1 prompted us to examine whether HFR1 can be modified with SUMO and further desumoylated by XopD<sub>Xcc8004</sub>. Here, the examination of the deduced amino acid sequence of HFR1 revealed a probable sumoylation site at lysine 72 (K72) in the consensus motif ΨKxE/D (where Ψ is a large and hydrophobic amino acid and x is any amino acid) (Fig. 6A). To address whether HFR1 can be modified with SUMO, an <i>in vitro</i> sumoylation assay was performed with <i>Arabidopsis</i> SAE1/SAE2 (SUMO-activating E1), SCE1 (SUMO-conjugating E2), AtSUMO1, and MBP-HFR1. Fig. 6B shows that a clear mobility shift of MBP-HFR1 was detected after incubation with the components of the <i>Arabidopsis</i> sumoylation cascade, and the shift was in a molecular mass consistent with mono-SUMO modification. Compared with MBP-HFR1, no mobility shift was detected for MBP-HFR1(K72A) in the <i>in vitro</i> sumoylation system (Fig. 6B). These results indicate that the K72 of HFR1 is the principal site
for SUMO conjugation. Next, we examined whether the sumoylated HFR1 can be desumoylated by XopD

\[ \text{XopD}_{8004} \text{Xcc}_{8004} \]

Fig. 6C shows that no sumoylated MBP-HFR1 was detected when XopD_{8004}, but not the catalytic mutant XopD_{8004(C355A)}, was present in the in vitro sumoylation reaction. These results indicate that XopD_{8004} catalyzes the SUMO hydrolysis from the K72 of HFR1.

\[ hfr1-201 \] increases resistance to \( Xcc_{8004} \)

Based on the interaction between HFR1 and XopD_{8004}, we propose that HFR1 may play a role in the plant immune response. To this end, we monitored the expression levels of genes involved in the SA-mediated defense-signaling network in hfr1–201 mutants and WT plants. Irrespective of treatment with or without 2 mM SA, we found that the levels of PR2, WRKY70, WIN3, EDS5, AIG1, PUB54, WRKY18, PR1, and WRKY51 transcripts are higher in the hfr1–201 mutant than in WT plants (Fig. 7A). We further investigated the growth of Xcc8004 WT and ΔXopD mutant strains in the hfr1–201 mutant. Compared with WT plants, multiplication of Xcc8004 and Xcc8004 ΔXopD strains was suppressed in the hfr1–201 mutant (Fig. 7B).
These results suggest that HFR1 is required for modulating the defense response in Arabidopsis, and the loss-of-function mutant in the HFR1 increases resistance to Xcc8004 spp.

**Discussion**

XopD is composed of an N-terminal domain, ERF-associated amphiphilic repression motifs, and a C-terminal SUMO protease domain. In XopD<sub>Xcv</sub>85–10 and XopD<sub>Xcc</sub>B100, the N-terminal domain has been suggested to play a role in specifying substrate recognition and modulating SUMO protease activity [16], [18]. However, XopD<sub>Xcc</sub>8004 was considered as a shorter version of XopD that does not contain an N-terminal domain [17], [19]. Using a transgenic approach, we found that possessing a C-terminal SUMO protease domain, the XopD<sub>Xcc</sub>8004-overexpressing transgenic plants showed morphological and molecular phenotypes similar to those of the mutants defective in the sumoylation machinery. In Arabidopsis, mutants defective in the sumoylation machinery, e.g., siz1 and sum1sum2 mutants, display localized programmed cell
death and elevated accumulation of SA, which contribute to the enhanced resistance against bacterial attacks [6], [7]. In this study, we showed that the expression of XopD<sub>8004</sub> in <i>Arabidopsis</i> elicited a lesion-mimic phenotype associated with increased expression of disease-response genes (Fig. 1B). Moreover, the multiplication of the <i>Xcc</i>8004 strain was suppressed in XopD<sub>8004</sub>-overexpressing transgenic plants (Fig. 2A), and <i>Xcc</i>8004<sup>Δ</sup>XopD displayed a higher bacterial growth rate than <i>Xcc</i>8004 (Fig. 2B). Different from XopD<sub>Xcv</sub> and XopD<sub>Xcc</sub>B100, which contain a complete N-terminal domain and are successful in suppressing anti-<i>Xcv</i> and anti-<i>Xcc</i>B100 immunities [16], [18], XopD<sub>Xcc</sub>8004 induced host defense responses to inhibit <i>Xcc</i>8004 growth. Although it is still unknown how the N-terminal domain modulates the SUMO protease activity of XopD, it is clear that impairment in the N-terminal domain of XopD not only loses the ability to suppress host immunity, but also elicits host defense responses.

Figure 7. <i>hfr1</i>–201 increases plant immunity against <i>Xcc</i>8004 spp. (a) <i>Arabidopsis</i> WT and <i>hfr1</i>–201 mutant plants treated with (8 hr) or without (0 hr) 2 mM SA were collected for total RNA extraction. The expression levels of genes involved in the SA-mediated defense signaling network were examined by qRT-PCR and normalized to EF1α. The relative expression levels of each gene in the WT plants without SA treatment were set at 1. (b) Bacterial growth in <i>Arabidopsis</i> WT and <i>hfr1</i>–201 mutant plants were measured to examine the effects of HFR1 on the resistance of <i>Arabidopsis</i> against <i>Xcc</i>8004 spp. Hand-infiltrated leaves were collected at the indicated times for measuring the <i>in planta</i> growth of bacterial populations. Statistically significant differences were determined using one-way ANOVA (* indicates <i>p</i> < 0.05).

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**XopD<sub>8004</sub> Elicits Host Defense Responses**
Based on the results that the catalytic mutant XopDXcc8004(C355A) was not able to elicit HR and plant immunity (Fig. 3A, B), we suggest that the activity of XopDXcc8004 in eliciting host defense responses is mainly dependent on the SUMO protease activity. Yet, the substrates of XopDXcc8004 functioning in plant defense responses remain largely unknown. Nevertheless, we identified that HFR1 was a potential nuclear substrate of XopDXcc8004 and could be modified by sumoylation (Figs. 5, 6). With the in vitro assay, we found that the sumoylated-residue K72 within the consensus sumoylation motif of HFR1 was desumoylated by XopDXcc8004 through the SUMO protease activity (Fig. 6B, C). Although the direct impact of SUMO protease activity of XopDXcc8004 on the function of HFR1 remains unknown, we observed that the loss-of-function mutation in HFR1 gene accelerated SA-mediated responses and increased the resistance against Xcc8004 (Fig. 7A, B). These results clearly indicate that HFR1 plays a role in repression of defense responses in plants.

HFR1 is an important regulator involved in photomorphogenesis and regulated by ubiquitin-mediated degradation through the coat protein complex I [27], [39], [40]. Because the highly unstable character of HFR1 [41], it is a technical challenge to provide a tight connection between XopDXcc8004 and HFR1 during infection. Thus, further experiments are required to demonstrate the function of sumoylation on the manipulation of the activity of HFR1. Sumoylation has been shown to play a role in mediating the transcriptional repression activity of many transcription factors [42–46]. Therefore, we do not exclude the possibility that the repression activity of HFR1 on defense responses requires a modification of SUMO. In fact, our data only explain a possible mechanism for XopDXcc8004-triggered host defense responses, and we do not exclude the possibility that XopDXcc8004 may have several targets in plants. Thus, we still observed a higher growth rate of Xcc8004 even in the hfr1–201 mutant (Fig. 7B).

Recently, Tan et al. (2014) reported that XopDXcc8004 triggers plant disease tolerance by targeting DELLA proteins [47]. In their study, they did not observe the significant difference in bacterial titers between Xcc8004 and Xcc8004 ΔXopD. This difference may be depending on the different experimental conditions used in our study. Here, a syringe infiltration method and a higher titer of bacterial suspension (2 × 10⁶ CFU mL⁻¹) were used. Nevertheless, our findings are not in conflict with the fact that XopDXcc8004 may act as a virulence-controlling factor by interfering with plant defense responses.

Recent studies on plant-pathogen interaction have provided new insights into fundamental cellular processes in plants [48], [49]. In this study, we identified a dual role of HFR1 in development and immunity. This finding indicates that HFR1 is required for the fine-tuning of the immune response, as well as contributes to our knowledge of the crosstalk between the light-signaling pathway and immune response.

Supporting Information

S1 Fig. Salicylic acid-dependent defense responses were elicited by the expression of XopDXcc8004 in Arabidopsis. (a) Trypan blue staining of two-week-old leaves of Arabidopsis XVE::XopDXcc8004 transgenic plants. Scale bar: 1 mm. (b) Translated products of XopDXcc8004 were examined by western blotting using a specific antibody against XopDXcc8004 and indicated by an arrow. Rubisco large subunit (RBCL) stained with coomassie brilliant blue served as a loading control. (c) The expression levels of genes involved in the SA-mediated defense signaling network were examined by qRT-PCR and normalized to EF1α. The relative expression levels of each gene in the DMSO control were set at 1.

(TIF)

S2 Fig. Transgenic plants harboring empty vector (XVE) did not show cell death phenotype upon β-estradiol treatment and elicited defense responses. (a) Trypan blue staining of two-
week-old leaves of Arabidopsis XVE transgenic plants. Scale bar: 1 mm. (b) Expression levels of genes involved in the SA-mediated defense signaling network were examined by qRT-PCR and normalized to EF1α. The relative expression levels of each gene in the DMSO control were set at 1.

(TIF)

S3 Fig. Salicylic acid-dependent defense responses elicited by the expression of XopDXcc8004 were inhibited by nahG. (a) Trypan blue staining of two-week-old leaves of Arabidopsis XVE::XopDXcc8004 / nahG transgenic plants. Scale bar: 1 mm. (b) Translated products of XopDXcc8004 were examined by western blotting using a specific antibody against XopDXcc8004 and indicated by an arrow. Rubisco large subunit (RBCL) stained with coomassie brilliant blue served as a loading control. (c) The expression levels of genes involved in the SA-mediated defense signaling network were examined by qRT-PCR and normalized to EF1α. The relative expression levels of each gene in the DMSO control were set at 1.

(TIF)

S4 Fig. XopDXcc8004(C355A) loses the activity for activating plant immunity. (a) Morphological examination and trypan blue staining of two-week-old leaves of Arabidopsis XVE::XopDXcc8004(C355A) transgenic plants. Scale bar: 1 mm. (b) Translated products of XopDXcc8004(C355A) were examined by western blotting using a specific antibody against XopDXcc8004 and indicated by an arrow. Rubisco large subunit (RBCL) stained with coomassie brilliant blue served as a loading control. (c) The expression levels of genes involved in the SA-mediated defense signaling network were examined by qRT-PCR and normalized to EF1α. The relative expression levels of each gene in the DMSO control were set at 1.

(TIF)

S5 Fig. Expression of XopDXcc8004 induces a long hypocotyl phenotype in Arabidopsis. (a, b) Average hypocotyl lengths of seedlings grown on medium containing DMSO (grey bars) and 20 μM β-estradiol (black bars). Statistically significant differences were determined using one-way ANOVA (** indicates p < 0.005).

(TIF)

S1 Table. Primer sequences for plasmid constructions and qRT-PCR.

(XLS)

S2 Table. Reads counting (mapped read sequence statistic) for Illumina sequencing data.

(XLS)

S3 Table. Differentially expressed genes with p < 0.001. Identification genes that were differentially expressed in Arabidopsis after XopDXcc8004 were induced by β-estradiol.

(XLS)

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Author Contributions

Conceived and designed the experiments: CMT JYY. Performed the experiments: CMT MYL PYY SHC YPH HL JYY. Analyzed the data: CMT JYY. Contributed reagents/materials/analysis tools: WLD. Wrote the paper: JYY.
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