The AvrB_AvrC Domain of AvrXccC of *Xanthomonas campestris* pv. *campestris* Is Required to Elicit Plant Defense Responses and Manipulate ABA Homeostasis

Yi-Ping Ho,¹ Choon Meng Tan,¹ Meng-Ying Li,¹ Hong Lin,¹ Wen-Ling Deng,² and Jun-Yi Yang^{1,3,4}

¹Institute of Biochemistry, National ChungHsing University, Taichung 40227, Taiwan; ²Department of Plant Pathology, National ChungHsing University, Taichung 40227, Taiwan; ³Institute of Biotechnology, National ChungHsing University, Taichung 40227, Taiwan; ⁴Agricultural Biotechnology Center, National ChungHsing University, Taichung 40227, Taiwan

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Plant disease induced by *Xanthomonas campestris* pv. *campestris* depends on type III effectors but the molecular basis is poorly understood. Here, AvrXccC₈₀₀₄ was characterized, and it was found that the AvrB_AvrC domain was essential and sufficient to elicit defense responses in an *Arabidopsis*-resistant ecotype (Col-0). An upregulation of genes in responding to the AvrB_AvrC domain of AvrXccC₈₀₀₄ was shown in a profile of host gene expression. The molecular changes were correlated with morphological changes observed in phenotypic and ultrastructural characterizations. Interestingly, the abscisic acid (ABA)-signaling pathway was also a prominent target for the AvrB_AvrC domain of AvrXccC₈₀₀₄. The highly elicited *NCED5*, encoding a key enzyme of ABA biosynthesis, was increased in parallel with ABA levels in AvrXccC₈₀₀₄ transgenic plants. Consistently, the *X. campestris* pv. *campestris* 8004 Δ avrXccC mutant was severely impaired in the ability to manipulate the accumulation of ABA and induction of ABA-related genes in challenged leaves. Moreover, exogenous application of ABA also enhanced the susceptibility of *Arabidopsis* to the *X. campestris* pv. *campestris* strains. These results indicate that the AvrB_AvrC domain of AvrXccC₈₀₀₄ alone has the activity to manipulate ABA homeostasis, which plays an important role in regulating the interactions of *X. campestris* pv. *campestris* and *Arabidopsis*.

Xanthomonas campestris pv. *campestris* is a gram-negative bacterium that is responsible for the black rot disease, one of the most destructive diseases on cruciferous plants (Williams 1980). This plant pathogen infects numerous important species of crucifers, including broccoli, mustard, cabbage, cauliflower, radish, and turnip, as well as the model plant *Arabidopsis thaliana* (Simpson and Johnson 1990). The successful infection requires protein secretion systems to deliver virulence factors into intracellular space or translocate directly into host cells (Büttner and Bonas 2010). One of the important secretion systems is the type III secretion system, which injects effector

proteins into host cell to interfere with host immunity response and plant cellular process to ensure bacterial multiplication and disease progression (White et al. 2009). In resistant plants, the virulence effectors might be recognized indirectly by host resistance (R) proteins through the virulence activities of effectors (DeYoung and Innes 2006). These recognition events often trigger the hypersensitive response, resulting in rapid localized cell death that restricts pathogen growth (Coll et al. 2011).

The outcomes of the plant–pathogen interactions depend on the balance between plant defense and pathogen counter-defense. The plant immune system contains two levels of defense responses: pathogen-associated molecular pattern (PAMP)-triggered immunity and effector-triggered immunity. PAMP-triggered immunity is a primary immune response activated by direct recognition of PAMPs, and effector-triggered immunity is a rapid immune response activated by indirect recognition of pathogen effectors (Dodds and Rathjen 2010). The signaling events induced by PAMPs or effectors are mainly regulated by plant hormones which activate successful defense responses against pathogen infections (Pieterse et al. 2009; Verhage et al. 2010). Three phytohormones, including salicylic acid (SA), jasmonic acid (JA), and ethylene, are known as primary signal molecules in regulating plant immune responses. Recently, other phytohormones, including abscisic acid (ABA), brassinosteroid, gibberellins, cytokinin, and auxin, also emerged as important components involved in plant immune responses (Bari and Jones 2009; Robert-Seilanianantz et al. 2007; Spoel and Dong 2008).

Pathogens have evolved various ways to interfere with plant immune responses. One of the striking ways is to produce plant hormones by pathogens themselves, such as *Agrobacterium tumefaciens*. *A. tumefaciens* is a crown gall disease bacterium that produces auxin to suppress host defense responses, thus enhancing host susceptibility to diseases (Dunoyer et al. 2006; Navarro et al. 2006). In addition, coronatine, a mimic molecule of JA, is synthesized by *Pseudomonas syringae* to affect JA homeostasis that antagonizes SA-mediated defense responses (Cui et al. 2005; Schulze-Lefert and Robatzek 2006). Another strategy is to modulate plant hormone signaling by type III effectors such as *P. syringae* AvrPtoB, which appears to be able to elevate ABA levels to overcome plant basal defense (de Torres-Zabala et al. 2007). Moreover, *P. syringae* AvrB appears to regulate JA signaling, which interferes with SA response and promotes plant susceptibility (Shang et al. 2006).

Corresponding author: J.-Y. Yang; E-mail: jyang@nchu.edu.tw

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Functional characterization of type III effectors is one of the key elements to understanding the molecular mechanisms of plant–pathogen interactions. In fact, the big challenge in systematic characterization of type III effectors is functional redundancy, in which mutations of individual effector genes usually do not affect the bacterial virulence or only have a subtle effect (Castañeda et al. 2005). In the past, different experimental approaches have been applied to study the mode of actions of individual effectors, which provided novel insights into the plant–pathogen interactions (Munkvold and Martin 2009; Salomon et al. 2011; Wilton and Desveaux 2010). Now, although more than 23 type III effectors were reported in *X. campestris* pv. *campestris* 8004 based on bioinformatic predications and experimental analyses, the molecular functions in pathogenesis of most effectors remain to be investigated (Büttner and Bonas 2010; Canonne et al. 2011; Jiang et al. 2008; Jiang et al. 2009; Wang et al. 2007; White et al. 2009; Xu et al. 2008).

Previous studies have shown that *X. campestris* pv. *campestris* type III effector AvrXccC₈₀₀₄ functions as an avirulence factor which is able to induce effector-triggered innate immunity and is responsible for disease resistance in *Arabidopsis* Col-0 (Rong et al. 2010; Wang et al. 2007). The resistance required *NDRI*,

RARI, and *SGT1b* to defend against *X. campestris* pv. *campestris* 8004; however, the responsible R protein for recognition of AvrXccC₈₀₀₄ has not been identified. AvrXccC₈₀₀₄ not only functions as an avirulence factor but also acts as a virulence factor. In *Arabidopsis* *rar1* and *sgt1b* mutants, *X. campestris* pv. *campestris* 8004-inoculated leaves showed higher pathogenicity responses than those inoculated with Δ avrXccC strain (Rong et al. 2010). AvrXccC₈₀₀₄ contains a conserved AvrB_AvrC domain between 113 to 438 amino acids (aa) which shared 61% similarity with the avirulence effector AvrB from *P. syringae* pv. *glycinea* (Gopalan et al. 1996; Wang et al. 2007). In this study, we used the inducible expression system to analyze the activities of AvrXccC₈₀₀₄ in *Arabidopsis* transgenic plants and focused on the functional studies of the AvrB_AvrC domain, which has not been characterized.

RESULTS

AvrB_AvrC domain of AvrXccC₈₀₀₄ is essential and sufficient to elicit defense responses in *Arabidopsis*.

Arabidopsis transgenic plants carried full-length AvrXccC₈₀₀₄ under the control of a β -estradiol-inducible promoter were

Fig. 1. Expression of AvrXccC₈₀₀₄ (110 to 440 amino acids [aa]) was sufficient to elicit cell death in *Arabidopsis*. **A**, Schematic representation of AvrXccC₈₀₀₄. The light-gray and dark-gray color boxes present the myristoylation site and AvrB_AvrC domain of AvrXccC₈₀₀₄, respectively. **B** through **F**, Phenotypes of 4-week-old *Arabidopsis* transgenic plants carried full length or a deletion series of AvrXccC₈₀₀₄ driven by XVE promoter. Scale bars: 5 mm. **G**, Leaf phenotypes of *XVE:AvrXccC8004* (1 to 440 aa) and *XVE:AvrXccC8004* (110 to 440 aa) transgenic plants. Scale bar: 5 mm. **H** and **I**, Trypan blue staining of transgenic plants expressing AvrXccC₈₀₀₄ (1 to 440 aa) and AvrXccC₈₀₀₄ (110 to 440 aa). Scale bars: 1 mm. **J**, Transcript levels of a deletion series of AvrXccC₈₀₀₄ in β -estradiol-treated transgenic plants. Stained rRNAs bands were used as a loading control.

generated (Fig. 1A). Compared with a dimethyl sulfoxide (DMSO) control, transgenic plants expressing AvrXccC₈₀₀₄ failed to grow after germination and developed chlorotic cotyledons upon 20 μ M β -estradiol treatment (Fig. 1B). Arrested growth associated with chlorotic cotyledons in *XVE:AvrXccC₈₀₀₄* (1 to 440 aa) transgenic plants may be accounted for the actions of AvrB_AvrC domain. To test this hypothesis, we introduced *XVE:AvrXccC₈₀₀₄* (110 to 440 aa) into *Arabidopsis* and analyzed the phenotypes in the presence of β -estradiol. Similar to *XVE:AvrXccC₈₀₀₄* (1 to 440 aa) transgenic plants, the expression of AvrXccC₈₀₀₄ (110 to 440 aa) resulted in arrested growth of seedlings and chlorosis in cotyledons (Fig. 1C). To define the subdomain responsible for developmental defects in *Arabidopsis*, AvrB_AvrC domain of AvrXccC₈₀₀₄ was divided into three parts: 110 to 237, 238 to 338, and 339 to 440 aa (Fig. 1A). Transgenic plants carrying *XVE:AvrXccC₈₀₀₄* (110 to 237aa), *XVE:AvrXccC₈₀₀₄* (238 to 338 aa), or *XVE:AvrXccC₈₀₀₄* (339 to 440 aa) were generated and analyzed in the presence of β -estradiol. Compared with the DMSO control, no developmental defects were found in individual transgenic lines (Fig. 1D to F). Further examination of the expression levels of AvrXccC₈₀₀₄ (110 to 237 aa), AvrXccC₈₀₀₄ (238 to 338 aa), and AvrXccC₈₀₀₄ (339 to 440 aa) illustrated that all transcripts were accumulated at levels even higher than those of AvrXccC₈₀₀₄ (1 to 440 aa) and AvrXccC₈₀₀₄ (110 to 440 aa) upon β -estradiol treatment (Fig. 1J). These results indicate that the intact AvrB_AvrC domain of AvrXccC₈₀₀₄ was essential to cause the developmental defects in *Arabidopsis*.

To further confirm whether the developmental defects shown in transgenic plants were due to the incompatible interactions between *Arabidopsis* and the AvrB_AvrC domain of AvrXccC₈₀₀₄, *XVE:AvrXccC₈₀₀₄* (1 to 440 aa) and *XVE:AvrXccC₈₀₀₄* (110 to 440 aa) transgenic plants were grown in soil and sprayed with 20 μ M β -estradiol after plants

were 4 weeks old. Compared with the DMSO control, chlorotic phenotypes were observed in the leaves of transgenic plants 2 days after spraying (Fig. 1G). Further examination using trypan blue staining confirmed that AvrXccC₈₀₀₄ (110 to 440 aa) could elicit cell death as well as full-length AvrXccC₈₀₀₄ (Fig. 1H and I). By contrast, no cell deaths were found in transgenic plants expressing AvrXccC₈₀₀₄ (110 to 237aa), AvrXccC₈₀₀₄ (238 to 338 aa), or AvrXccC₈₀₀₄ (339 to 440 aa) (Supplementary Fig. S1). These results suggest that the AvrB_AvrC domain of AvrXccC₈₀₀₄ is sufficient to elicit the incompatible interactions which result in cell death phenotype.

Ultrastructural characterizations of transgenic plants expressing AvrB_AvrC domain of AvrXccC₈₀₀₄.

To understand the *Arabidopsis* cellular responses to the actions of the AvrB_AvrC domain of AvrXccC₈₀₀₄ in detail, leaf sections of *XVE:AvrXccC₈₀₀₄* (110 to 440 aa) transgenic plants were examined. Under light microscopy, differences were observed in cell integrity of *XVE:AvrXccC₈₀₀₄* (110 to 440 aa) transgenic plants between DMSO and β -estradiol treatments (Fig. 2A and E). For DMSO treatment, cells were arranged with a proper space (Fig. 2A). The roughly pill-shaped chloroplasts, which were tightly connected with each other and pushed against the cell periphery, were frequently observed (Fig. 2A). By contrast, after β -estradiol treatment, cells were separated by a widened intercellular space, and the chloroplasts were not only flat and fewer but also distant from the cell periphery (Fig. 2E). These phenomena can indicate the loss of turgor pressure in vacuoles caused by plasmolysis (Hatsugai et al. 2009). We further examined the exchanges of subcellular ultrastructure using transmission electron microscopy (TEM) upon β -estradiol treatment (Fig. 2F to I). Compared with DMSO control (Fig. 2B and C), the

Fig. 2. Cellular responses in *Arabidopsis* transgenic plants expressing AvrXccC₈₀₀₄ (110 to 440 amino acids). Rosette leaves from 5-week-old *Arabidopsis* transgenic plants were collected for embedding with resin. **A** and **E**, Semi-thin sections (1 μ m) were stained with toluidine blue for general view of morphological changes by light microscopy. **B** through **D** and **F** through **I**, For ultrastructural characterizations, ultrathin sections (80 nm) were processed and viewed by transmission electron microscopy. Images were collected and compared between β -estradiol treatment and dimethyl sulfoxide (DMSO) control. Visible organelles were indicated as follows: cw, cell wall; cyt, cytosol; pm, plasma membrane; vm, vacuolar membrane; ch, chloroplast; ob, oil body; sg, starch granule; asterisks, intercellular spaces; arrows, detached chloroplasts; arrowheads, vesicles.

reduced number of starch granules and presence of oil droplets (plastoglobuli) were frequently observed (Fig. 2F and G), which were usually accompanied by cell death under stress conditions (Mizuno et al. 2010; Wright et al. 2009). Moreover, plasma membranes became rippled and associated with small vesicles (Fig. 2H and I) compared with the DMSO control (Fig. 2D). Consistent with the morphological changes shown in Figure 1C and I, the ultrastructural characterizations confirm that the AvrB_AvrC domain of AvrXccC₈₀₀₄ is important for the incompatible interactions in *Arabidopsis*.

In addition, leaf sections of XVE:AvrXccC₈₀₀₄ (1 to 440 aa) transgenic plants were also examined. Similar to the ultrastructural changes observed in XVE:AvrXccC₈₀₀₄ (110 to 440 aa) transgenic plants, the widened intercellular space, detached chloroplasts with slim shape, and presence of oil droplets together with twisted plasma membranes and small vesicles were also observed in XVE:AvrXccC₈₀₀₄ (1 to 440 aa) transgenic plants after β -estradiol treatment (Supplementary Fig. S2A through I).

RNA-Seq profiling analysis using high-throughput next-generation sequencing.

To correlate the phenotypic alterations with molecular changes, transcriptome data of XVE:AvrXccC₈₀₀₄ (110 to 440 aa) transgenic plants upon DMSO and β -estradiol treatments were analyzed. After quality trim, 24.9 million reads and 24.3 billion bases were generated by Illumina 100-bp single-end sequencing from two cDNA libraries (Supplementary Table S1). Among them, 89% of the sequenced reads from DMSO treatment were mapped to 20,845 *Arabidopsis* genes and 86% of the sequenced reads from β -estradiol treatment were mapped to 21,102 *Arabidopsis* genes (Supplementary Table S2). The expression levels of mapped genes from two samples were quantified and converted to PRKM values based on sequenced reads. Stable housekeeping genes (Gutierrez et al. 2008), including *TUB9*, *ACT7*, *ACT8*, *UBQ5*, and *EF1 α* , were used as reference genes. The change of reads per kilobase of exon model per million mapped reads (RPKM) values for the reference genes ranged from 0.91-fold (*ACT8*) to 1.11-fold (*ACT7*), indicating that the expression levels of reference genes were similar across different treatments (Supplementary Table S3). These results suggest that the RNA-Seq data is reliable and can be used for further analysis.

To identify differentially expressed genes in XVE:AvrXccC₈₀₀₄ (110 to 440 aa) transgenic plants upon β -estradiol treatment, differential expression analysis using the DESeq method (Anders and Huber 2010) was performed. Compared with DMSO treatment, 771 genes with $P < 0.05$ were identified after β -estradiol treatment. Among them, 693 genes were upregulated and 78 genes were downregulated. To narrow down the differentially expressed genes, a criteria with $P < 0.01$ and $\log_2$ (fold change) > 3.0 or < -3.0 was selected. As a result, 337 genes were identified. Among them, 324 genes were upregulated and 13 genes were downregulated (Supplementary Table S4). Using Gene Ontology (GO) analysis to classify the 324 differentially expressed genes revealed that several terms, including cellular process, response to stimulus, metabolic process, cell, catalytic activity, and binding, were highlighted in individual categories (Supplementary Fig. S3). In all, 98 of 337 differentially expressed genes were mapped to the “response to stimulus” term (Supplementary Table S5), which suggested that *Arabidopsis* could respond to the activities of AvrXccC₈₀₀₄ (110 to 440 aa) and regulated gene expression for cellular or behavioral stimulations. Under the response to stimulus term, 38 genes were further mapped to the “defense response” term and 31 genes were further mapped to the “response to other organism” term, which indicated that the induction of

AvrXccC₈₀₀₄ (110 to 440 aa) strikingly triggered the plant immune system related to pathogen infection.

Multiple plant defense systems were triggered by AvrXccC₈₀₀₄ (110 to 440 aa).

Functional annotation on the 337 differentially expressed genes ($P < 0.01$) revealed that multiple plant defense systems were triggered in XVE:AvrXccC₈₀₀₄ (110 to 440 aa) transgenic plants upon β -estradiol treatment. Remarkably, an upregulation of genes involved in the SA-mediated defense signaling network (Lu 2009) was shown in responding to AvrXccC₈₀₀₄ (110 to 440 aa). The transcriptome profiling data showed that pathogenesis-related (PR)1 and PR2, which are standard markers for SA-mediated defense, were induced 1,182- and 100-fold, respectively (Table 1). At the same time, transcript levels of genes involved in SA biosynthesis (*EDS5*, *SID2*, *SARD1*, and *CBP60g*) (Nawrath et al. 2002; Wang et al. 2011b; Wildermuth et al. 2001) as well as SA accumulation and signaling (*ALDI*, *WIN3*, *PAD4*, *FMO1*, *NUDX6*, and *PDL5*) (Ishikawa et al. 2010; Lee et al. 2011; Mishina and Zeier 2006; Song et al. 2004; Wang et al. 2011a; Zhou et al. 1998) were highly elevated (Table 1). Among these genes, *SID2*, which encodes an isochorismate synthase that produces an immediate precursor of SA, was induced 26-fold; and *PAD4*, which encodes a lipase-like protein that interacts directly with *EDS1* (Feys et al. 2001), an essential element for hypersensitive response, was induced 31-fold. For downstream signaling, WRKY transcription factors (*WRKY51*, *WRKY72*, *WRKY8*, *WRKY 38*, and *WRKY50*), which were classified into the defense response term, had higher expression levels (Table 1). In addition, other plant defense response-associated genes, including *DOX1*, *AIG1*, *CML43*, *PARG2*, *UGT85A1*, *TRX5*, *CDA1*, *SDR3*, and *LOX1* (Adams-Phillips et al. 2010; Carviel et al. 2009; Chiasson et al. 2005; Hwang et al. 2011; Laloi et al. 2004; Reuber and Ausubel 1996; Vicente et al. 2012) were also highly expressed (Table 1). Among them, *DOX1* and *LOX1*, which encode key enzymes for oxylipins that play important roles in plant defense, were induced 1,536-fold and 15-fold, respectively.

Here, in an *Arabidopsis* resistant ecotype (Col-0), a group of genes which encode Toll-interleukin-1 receptor (TIR) domain-containing proteins, including TIR adaptor proteins (At5g38344, At4g19925, At1g57630, and At2g20142) and TIR nucleotide-binding site leucine-rich repeat (NBS-LRR) proteins (At5g38350, At4g11340, At5g18350, and At3g04220), were highly upregulated by AvrXccC₈₀₀₄ (110 to 440 aa) (Table 2). However, several genes, including *BONI*, *BON3*, *BAP1*, *BAP2*, and *KTII* (Li et al. 2008; Li et al. 2009; Yang et al. 2006a; Yang et al. 2006b; Yang et al. 2007), which act as negative regulators to repress R-mediated defense responses, were also highly elevated by AvrXccC₈₀₀₄ (110 to 440 aa) (Table 2). Thus, AvrXccC₈₀₀₄ (110 to 440 aa) may be able to manipulate R-gene-mediated responses through its virulence activities to counteract plant defense responses.

Expression of AvrXccC₈₀₀₄ (110 to 440 aa) interfered with the ABA-signaling responses.

In addition to defense response mediated by the SA-signaling pathway, the ABA-signaling pathway was also found to be a prominent target for AvrXccC₈₀₀₄ (110 to 440 aa) (Table 3). The upregulated genes were involved in ABA biosynthesis, accumulation, and signaling transduction. For ABA biosynthesis, *NCED5*, which encodes a 9-*cis*-epoxycarotenoid dioxygenase that cleavages the precursor of ABA (Frey et al. 2012), was induced 151-fold. For ABA accumulation and signaling, *PDR12*, which encodes an ABC transporter that mediates cellular uptake of ABA (Kang et al. 2010), was induced 85-fold; *SAG113*, which encodes a PP2C-type phosphatase that acts as a regulator

of the ABA signaling pathway (Zhang et al. 2012), was induced 154-fold. For downstream signaling, *ABO3* and *NAC019*, which encode WRKY and NAC transcription factors (Jensen et al. 2010; Ren et al. 2010), were induced 32- and 20-fold, respectively. In addition, other ABA response-associated genes, includ-

ing *SDR4*, *PNP-A*, *5PTASE11*, *SAP12*, *ARSK1*, *RLP41*, *UGT76B1*, *CRK36*, *LECRKA4.2*, and *ARCK1* (de Torres-Zabala et al. 2007; Ercetin and Gillaspay 2004; González-Guzmán et al. 2002; Hwang and Goodman 1995; Tanaka et al. 2012; von Saint Paul et al. 2011; Wang et al. 2008; Wang et al. 2011c; Xin et al.

Table 1. Classification and annotation of the differentially expressed genes (P value < 0.01) involved in plant defense system

Category, name	AGI number ^b	Base mean ^a		Fold change ^c	Functional annotations
		β -estradiol	DMSO		
PR genes ^d					
<i>PR1</i>	At2g14610	113,377.16	95.893	1,182.3	Pathogenesis-related 1
<i>PR2</i>	At3g57260	2,443.0322	24.506	99.691	Pathogenesis-related 2
SA biosynthetic ^e					
<i>EDS5</i>	At4g39030	3,487.6326	120.4	28.967	Enhanced disease susceptibility 5
<i>SARD1</i>	At1g73805	6,853.2545	295.14	23.221	SAR deficient 1
<i>SID2</i>	At1g74710	4,121.1504	157.69	26.134	Salicylic acid induction deficient 2
<i>CBP60g</i>	At5g26920	3,950.3353	263.17	15.01	Calmodulin-binding protein 60g
SA accumulation ^f					
<i>ALD1</i>	At2g13810	3,410.672	33.03	103.26	AGD2-like defense response protein 1
<i>WIN3</i>	At5g13320	11,246.583	125.73	89.453	HopW1-1-interacting 3
<i>NUDX6</i>	At2g04450	7,305.6331	85.238	85.708	Nudix hydrolase homolog 6
<i>FMO1</i>	At1g19250	8,314.5688	23.441	354.71	Flavin-dependent monooxygenase 1
<i>PAD4</i>	At3g52430	11,497.174	375.05	30.655	Phytoalexin deficient 4
<i>PDLP5</i>	At1g70690	1,842.3635	98.024	18.795	Plasmodesmata-located protein 5
Downstream ^g					
<i>WRKY51</i>	At5g64810	1,115.9298	21.31	52.368	WRKY transcription factor
<i>WRKY72</i>	At5g15130	652.28867	15.982	40.813	WRKY transcription factor
<i>WRKY8</i>	At5g46350	1,080.2651	31.964	33.796	WRKY transcription factor
<i>WRKY38</i>	At5g22570	649.47303	33.03	19.663	WRKY transcription factor
<i>WRKY50</i>	At5g26170	422.34518	17.048	24.774	WRKY transcription factor
Defense response ^h					
<i>DOX1</i>	At3g01420	11,457.755	7.4584	1,536.2	Dioxygenase 1
<i>ATG1</i>	At1g33960	38,678.372	200.31	193.09	AvrRpt2-induced gene 1
<i>CML43</i>	At5g44460	136.089	1.0655	127.73	Calmodulin like 43
<i>PARG2</i>	At2g31865	1,982.2067	44.75	44.295	Poly (ADP-ribose) glycohydrolase 2
<i>UGT85A1</i>	At1g22400	12,727.607	438.98	28.994	UDP-glucosyl transferase 85A1
<i>TRX5</i>	At1g45145	7,811.5087	312.19	25.022	Cytosolic thioredoxin
<i>CDA1</i>	At2g19570	1,130.008	58.601	19.283	Cytidine deaminase 1
<i>SDR3</i>	At2g47130	1,194.7676	76.715	15.574	Short-chain dehydrogenase/reductase 3
<i>LOX1</i>	At1g55020	2,132.3739	142.77	14.935	Lipoxygenase 1

^a Base mean: the number of reads divided by the size factor (normalization constant) of sample. DMSO = dimethyl sulfoxide

^b AGI = *Arabidopsis* Genome Initiative.

^c Fold change = β -estradiol base mean/DMSO base mean.

^d PR = pathogenesis related.

^e Salicylic acid (SA) biosynthetic process.

^f SA accumulation and signaling.

^g Putative downstream signaling components.

^h Defense response-associated genes.

Table 2. Classification and annotation of the differentially expressed genes (P < 0.01) involved in resistance- or cell-death- related responses

Category, name	AGI number ^b	Base mean ^a		Fold change ^c	Functional annotations ^d
		β -estradiol	DMSO		
Resistance response ^e					
	At5g38344	172.69225	1.0654792	162.0794	TIR domain family
	At4g19925	119.1952	1.0654792	111.87	TIR domain family
	At1g57630	2,761.1989	58.601355	47.11835	TIR domain family
	At2g20142	1,026.7681	47.946563	21.41484	TIR domain family
	At5g38350	573.4509	1.0654792	538.2094	Disease resistance protein (TIR-NBS-LRR class)
	At4g11340	246.83729	1.0654792	231.6679	Disease resistance protein (TIR-NBS-LRR class)
	At5g18350	134.21191	1.0654792	125.9639	Disease resistance protein (TIR-NBS-LRR class)
	At3g04220	309.7198	12.78575	24.22383	Disease resistance protein (TIR-NBS-LRR class)
Cell death					
<i>BAP2</i>	At2g45760	2,243.1222	2.1309584	1,052.635	Similar to BONZAI1-binding protein (BAP1)
<i>KT11</i>	At1g73260	4,257.2394	10.654792	399.561	Kunitz trypsin inhibitor 1 (KT11)
<i>BON1</i>	At5g61900	2,020.6871	106.54792	18.96505	Encodes a copine-like protein
<i>BON3</i>	At1g08860	101.36284	2.1309584	47.56679	Encodes a copine-like protein
<i>BAP1</i>	At3g61190	1,154.4102	51.143001	22.5722	BON ASSOCIATION PROTEIN 1

^a Base mean: the number of reads divided by the size factor (normalization constant) of sample. DMSO = dimethyl sulfoxide.

^b AGI = *Arabidopsis* Genome Initiative.

^c Fold change = β -estradiol base mean/DMSO base mean.

^d TIR = Toll-interleukin-resistance, NBS-LRR = nucleotide-binding site leucine-rich repeat.

2009), were also highly expressed. Among them, *SDR4*, which encodes a short-chain dehydrogenase/reductase that may participate in ABA synthesis, was induced 128-fold; *PNP-A*, which encodes a plant natriuretic peptide that modulates the effects of ABA, was induced 88-fold; and *UGT76B1*, which encodes a glucosyltransferase that attenuates SA-mediated defense system, was found to contain a ABA response element in the promoter. These results suggest that *X. campestris* pv. *campestris* 8004 may interfere plant ABA signaling transduction by *AvrXccC*₈₀₀₄.

To validate the RNA-Seq profiling on the differentially expressed genes induced by *AvrXccC*₈₀₀₄ (110 to 440 aa), quantitative real-time reverse-transcription polymerase chain reaction (qRT-PCR) was used. Nine genes involved in plant defense responses, especially in the SA-signaling pathway, including biosynthetic process, accumulation, signaling transduction, and downstream response, were evaluated using *EF1α* as a reference gene. *PRI*, *EDS5*, *SID2*, *WIN3*, *FMO1*, *PAD4*, *WRKY51*, *DOX1*, and *AIG1* were upregulated after β-estradiol treatment (Fig. 3A). At the same time, *NCED5*, *SAG113*, *PDR12*, *ABO3*, *NAC019*, *PNP-A*, and *SAP12*, which are involved in ABA biosynthesis, ABA signaling transduction, and ABA regulated response, were also evaluated using *EF1α* as a reference gene. Similar to the examined genes involved in plant defense responses, all ABA-signaling response genes were upregulated after β-estradiol treatment (Fig. 3B). Here, our qRT-PCR results were consistent with the high-throughput gene expression profiling produced by next-generation sequencing.

To further examine whether *AvrXccC*₈₀₀₄ (1 to 440 aa) triggered molecular responses similar to *AvrXccC*₈₀₀₄ (110 to 440 aa), all of the above-validated genes were investigated again by qRT-PCR using RNA samples extracted from *XVE:AvrXccC*₈₀₀₄ (1 to 440 aa) transgenic plants. Compared with the DMSO control, all examined genes involved in plant defense responses and ABA-signaling responses were upregulated upon β-estradiol treatment (Fig. 3C and D). However, the relative expression ratios of examined genes induced by *AvrXccC*₈₀₀₄ (1 to 440 aa) were not as high as those induced by *AvrXccC*₈₀₀₄ (110 to 440 aa), although the transcript levels and protein amounts

of *AvrXccC*₈₀₀₄ (110 to 440 aa) were only slightly higher than those of *AvrXccC*₈₀₀₄ (1 to 440 aa) in *Arabidopsis* after β-estradiol treatment (Fig. 3E and F).

***AvrXccC* of *X. campestris* pv. *campestris* 8004 is required to manipulate ABA biosynthesis and signaling.**

The highly induced *NCED5*, which encodes a key regulatory enzyme in the ABA biosynthetic process, prompted us to examine whether *AvrXccC*₈₀₀₄ could manipulate the accumulation of ABA. ABA amounts were induced 2.4- and 3.0-fold upon β-estradiol treatment in *XVE:AvrXccC*₈₀₀₄ (1 to 440 aa) and *XVE:AvrXccC*₈₀₀₄ (110 to 440 aa) transgenic plants, respectively (Fig. 4A). We further examined the ABA amounts and ABA-related gene expression levels in *X. campestris* pv. *campestris* 8004 wild-type (WT)- and *ΔavrXccC*-challenged leaves. The *ΔavrXccC* mutant was severely impaired in the ability to manipulate the accumulation of ABA in challenged leaves (Fig. 4B). Consistently, the expression levels of *NCED5*, *SAG113*, *ABO13*, and *NAC019* involved in ABA biosynthesis, accumulation, and signaling were dramatically reduced in leaves challenged by the *ΔavrXccC* mutant (Fig. 4C).

Exogenous application of ABA enhances the multiplication of *X. campestris* pv. *campestris* 8004.

In order to test the effect of exogenous ABA on the growth of *X. campestris* pv. *campestris* 8004, ABA was sprayed on *Arabidopsis* leaves 24 h before bacterial inoculation. Followed ABA pretreatment, *X. campestris* pv. *campestris* 8004 strains, including the WT and *ΔavrXccC* mutant, were inoculated into *Arabidopsis* leaves. Exogenous application of ABA increased the populations of *X. campestris* pv. *campestris* 8004 strains by more than two orders of magnitude at 3 days post inoculation compared with mock (0.2% ethanol) treatment (Fig. 5A and B). Thus, the exogenous application of ABA allowed *X. campestris* pv. *campestris* 8004 strains to partially overcome host defense responses. These results indicate that ABA is a key factor in regulating the interactions of *X. campestris* pv. *campestris* and *Arabidopsis*.

Table 3. Classification and annotation of the differentially expressed genes ($P < 0.01$) involved in abscisic acid (ABA) responses

Category, name	AGI number ^b	Base mean ^a		Fold change ^c	Functional annotations
		β-estradiol	DMSO		
ABA biosynthetic ^d					
<i>NCED5</i>	At1g30100	160.491	1.06548	150.63	9-cis-Epoxycarotenoid dioxygenase
ABA accumulation ^e					
<i>SAG113</i>	At5g59220	492.736	3.19644	154.15	Senescence associated gene 113
<i>PDR12</i>	At1g15520	12,631.9	148.102	85.292	Pleiotropic Drug resistance 12
Downstream ^f					
<i>ABO3</i>	At1g66600	370.725	11.7203	31.631	ABA Overly sensitive mutant 3
<i>NAC019</i>	At1g52890	1,615.24	79.9109	20.213	NAC transcription factor
ABA response ^g					
<i>SDR4</i>	At3g29250	409.206	3.19644	128.02	Short-chain dehydrogenase/reductase 4
<i>PNP-A</i>	At2g18660	2,987.39	34.0953	87.619	Plant natriuretic peptide A
<i>5PTASE11</i>	At1g47510	305.966	3.19644	95.721	Inositol polyphosphate 5-phosphatase 11
<i>SAP12</i>	At3g28210	838.121	21.3096	39.331	Stress-associated protein 12
<i>ARSK1</i>	At2g26290	222.435	6.39288	34.794	<i>Arabidopsis</i> root-specific kinase 1
<i>RLP41</i>	At3g25010	1,590.83	55.4049	28.713	Receptor like protein 41
<i>UGT76B1</i>	At3g11340	1,755.08	67.1252	26.146	Glucosyltransferase
<i>CRK36</i>	At4g04490	2,588.51	122.53	21.125	Cysteine-rich receptor like protein kinase 36
<i>LECKRA4.2</i>	At5g01550	1,001.43	54.3394	18.429	Lectin receptor kinase A4.2
<i>ARCK1</i>	At4g11890	905.696	42.6192	21.251	ABA- and osmotic stress-inducible receptor-like cytosolic kinase 1

^a Base mean: the number of reads divided by the size factor (normalization constant) of sample. DMSO = dimethyl sulfoxide.

^b AGI = *Arabidopsis* Genome Initiative.

^c Fold change = β-estradiol base mean/DMSO base mean.

^d ABA biosynthetic process.

^e ABA accumulation and signaling.

^f Downstream signaling components.

^g ABA response-associated genes.

DISCUSSION

In *Arabidopsis*, the effects of ABA in regulating plant-pathogen interactions have been intensively studied in recent years (Asselbergh et al. 2008). In *P. syringae*, it has been demonstrated that the manipulations of host ABA biosynthesis and response machinery were mainly controlled by the virulence effectors (de Torres-Zabala et al. 2007). Here, in *X. campestris* pv. *campestris*, AvrXccC₈₀₀₄ not only presented its avirulence activity to trigger plant defense responses but also possessed its virulence activity to manipulate the components involved in the ABA signaling pathway, leading to an increase of ABA concentrations and disturbance of ABA signaling responses. Our data suggest that the increase of ABA levels was caused by the highly expressed *NCED5*. *NCED5* not only encodes a key enzyme involved in ABA biosynthesis (Frey et al. 2012) but also plays an important role in regulating plant defense

responses because the *cds2-1D* mutant, an activation-tagged line with highly expressed *NCED5*, showed the enhanced disease susceptibility phenotype (Fan et al. 2009). As a result, AvrXccC₈₀₀₄ may attenuate host defense responses by elevating ABA levels. This hypothesis was supported by our findings on exogenous application of ABA, which enhanced the multiplication of *X. campestris* pv. *campestris* in *Arabidopsis*. Recently, Choi and Hwang (2011) also showed that ABR1, a negative regulator of ABA biosynthesis, was required for the resistant pepper to protect against *X. campestris* pv. *vesicatoria*. Taken together, it is clear that ABA functions as a negative signaling molecule to suppress host immunity required to protect against *Xanthomonas* spp. Although ABA plays an important role in modulating the pathogenicity of *Xanthomonas* spp., the molecular mechanisms of ABA actions in plant defense pathways are still unclear and warrant further investigation.

Fig. 3. Quantitative reverse-transcription polymerase chain reaction (qRT-PCR) validation of differentially expressed genes. mRNA levels of genes involved in **A** and **C**, plant defense responses and **B** and **D**, abscisic acid (ABA) signaling responses were normalized with *EF1 α* , and the relative expression levels in a dimethyl sulfoxide (DMSO) control were set at 1 for each gene. **E**, Relative expression levels of *AvrXccC*₈₀₀₄ (110 to 440 amino acids [aa]) and *AvrXccC*₈₀₀₄ (1 to 440 aa) were examined by qRT-PCR and **F**, the translated products of those transcripts were examined by Western blotting using specific polyclonal antibody against *AvrXccC*₈₀₀₄ (110 to 440 aa). Anti- α -tubulin was used for loading control. Average and standard error values of three independent repeats are given.

In *Arabidopsis*, there are 149 NBS-LRR-encoding genes that can be divided into two distinct groups: TIR-NBS-LRR and coiled-coil motif (CC)-NBS-LRR, which play an important role in pathogen sensing and host defense (DeYoung and Innes 2006; Meyers et al. 2003). Early studies demonstrated that TIR-NBS-

Fig. 4. AvrXccC of *Xanthomonas campestris* pv. *campestris* 8004 is required to manipulate abscisic acid (ABA) biosynthesis and signaling. **A**, Quantification of ABA levels in *Arabidopsis* plants expressing AvrXccC₈₀₀₄ (1 to 440 amino acids [aa]) and AvrXccC₈₀₀₄ (110 to 440 aa). ABA levels in *Arabidopsis* transgenic plants were determined by a phytodetek ABA enzyme-linked immunosorbent assay kit and the fold changes in ABA levels after β -estradiol treatment were shown in boxes. **B**, Quantification of ABA levels in *X. campestris* pv. *campestris* 8004 wild-type (WT)- and Δ avrXccC-challenged leaves. ABA levels in challenged leaves were determined at 3 days post inoculation (dpi). **C**, Examination of ABA-related gene expression levels in *X. campestris* pv. *campestris* 8004 WT- and Δ avrXccC-challenged leaves by quantitative reverse-transcription polymerase chain reaction. Average and standard error values of three independent repeats are given.

LRR proteins and CC-NBS-LRR proteins mounted different signaling pathways for R-mediated resistant responses (Aarts et al. 1998; Glowacki et al. 2011). For the TIR-NBS-LRR protein-mediated signaling pathway, EDS1 played a key role as a mediator which interacted with PAD4 to regulate the levels of SA (Feys et al. 2001). The activity of EDS1 was also regulated by a monooxygenase, FMO1 (Bartsch et al. 2006). For CC-NBS-LRR protein-mediated signaling pathways, NDR1 played a key role as a mediator to trigger resistance responses. The RNA-Seq profiling assay obtained here revealed that only genes encoding TIR-NBS-LRR proteins were highly upregulated, which suggests that the AvrB_AvrC domain of AvrXccC₈₀₀₄ may trigger EDS1-mediated resistance responses. Moreover, the highly expressed *PAD4* and *FMO1* induced by AvrXccC₈₀₀₄ (110 to 440 aa) further support the idea. Because no R protein responsible for the type III effectors of *X. campestris* pv. *campestris* 8004 was identified thus far, it will be interesting to isolate the corresponding R gene, which should provide invaluable insights into the molecular mechanism of the effector-triggered immunity signaling pathway in *Arabidopsis*.

In this study, we showed that the intact AvrB_AvrC domain of AvrXccC₈₀₀₄ is essential and sufficient to elicit defense responses, because the dissection of AvrXccC₈₀₀₄ (110 to 440 aa) into three subdomains—AvrXccC₈₀₀₄ (110 to 237aa), AvrXccC₈₀₀₄ (238 to 338 aa), and AvrXccC₈₀₀₄ (339 to 440 aa)—fully disrupts its avirulence functions. These results suggest that the functional activities of the AvrB_AvrC domain of AvrXccC₈₀₀₄ are necessary to be present for the recognition by the host R-mediated defense system more than structural or

Fig. 5. Effect of exogenous application of abscisic acid (ABA) on the growth of *Xanthomonas* spp. in wild-type (WT) *Arabidopsis*. Multiplication of **A**, *Xanthomonas campestris* pv. *campestris* 8004 WT and **B**, Δ avrXccC mutant strains on *Arabidopsis* plants sprayed with ABA. Exogenous 100 μ M ABA or 0.2% ethanol were applied on *Arabidopsis* 24 h before bacterial inoculation. Hand-infiltrated leaves were collected at indicated times for measuring in planta growth of bacterial populations. Average and standard error values of three independent repeats are given.

sequential presentations. Recently, with bioinformatic predictions, it has been suggested that AvrXccC contains a presumable fido domain, which may be able to modify host proteins by AMPylation (Kinch et al. 2009). However, it remains to be determined whether AvrXccC₈₀₀₄ can possess its functional activities through AMPylation to modify host proteins which, in turn, modulate host defense responses.

Previous studies showed that the N-terminal myristoylation of AvrXccC₈₀₀₄ was essential for membrane localization and the myristoylation site mutation dramatically destroyed the avirulence activity of AvrXccC₈₀₀₄ (Wang et al. 2007). In this study, we found that overexpression of AvrXccC₈₀₀₄ (110 to 440 aa) as well as AvrXccC₈₀₀₄ (1 to 440 aa) was able to trigger plant resistance responses and caused cell death in *Arabidopsis*. To clarify the subcellular localization of AvrXccC₈₀₀₄ (110 to 440 aa) in *Arabidopsis*, C-terminal yellow fluorescent protein (YFP)-tagged AvrXccC₈₀₀₄ (110 to 440 aa) was expressed transiently in mesophyll protoplast. Similar to the localization pattern of full-length AvrXccC₈₀₀₄ with a deleted N-terminal myristoylation site (Wang et al. 2007), the AvrXccC₈₀₀₄ (110 to 440 aa) lost the ability for plasma membrane targeting (Supplementary Fig. S4). However, instead of even distribution in onion epidermis cells for myristoylation site-mutated AvrXccC₈₀₀₄-YFP (Wang et al. 2007), the fluorescence signals of AvrXccC₈₀₀₄ (110 to 440 aa)-YFP were observed as cytoplasmic foci in *Arabidopsis* protoplasts. Although the cytoplasmic localization of AvrXccC₈₀₀₄ (110 to 440 aa) did not abolish its avirulence activity, it is still unclear how AvrXccC₈₀₀₄ (110 to 440 aa) was recognized and triggered resistance responses. Hence, the identification of AvrXccC₈₀₀₄ targets or host R proteins will clarify how the cytoplasmic foci formed by AvrXccC₈₀₀₄ (110 to 440 aa) responsible for the resistance responses.

Conclusion.

X. campestris pv. *campestris* type III effector AvrXccC₈₀₀₄ has been characterized as a bifunctional effector, which induced strong defense responses in WT *Arabidopsis* Col-0 and acted as virulence factor in *rar1* and *sgt1b* mutants (Rong et al. 2010). In this study, we further characterized the functions of AvrXccC₈₀₀₄ using *Arabidopsis* protoplast and transgenic approaches. We found that the AvrB_AvrC domain of AvrXccC₈₀₀₄ was essential and sufficient to elicit defense responses, because both AvrXccC₈₀₀₄ (1 to 440 aa) and AvrXccC₈₀₀₄ (110 to 440 aa) induced similar physiological phenotypes as well as molecular phenotypes. Remarkably, we showed that the manipulation of ABA biosynthesis and response machinery by AvrXccC₈₀₀₄ is an important strategy to attenuate host defense responses. However, as an avirulence factor, AvrXccC₈₀₀₄ elicited strong R-mediated resistance responses, mainly through the SA-signaling pathway, which suppressed the antagonistic functions of ABA activated by the virulence activities of AvrXccC₈₀₀₄. As a result, cell death, the typical outcome of hypersensitive response, was observed in both *XVE:AvrXccC₈₀₀₄* (1 to 440 aa) and *XVE:AvrXccC₈₀₀₄* (110 to 440 aa) transgenic plants upon β -estradiol treatment.

MATERIALS AND METHODS

Plant materials, growth conditions, and β -estradiol treatments.

Arabidopsis ecotype Col-0 was used for protoplast preparation, transgenic plant generation, and *Xanthomonas* spp. inoculation. Plants were grown at 21°C in a semi-controlled walk-in chamber with a photoperiod of 16 and 8 h at 8,000 lux for *Agrobacterium* transformation, and a photoperiod of 12 and 12 h at 8,000 lux for protoplast preparation and *Xanthomonas*

spp. inoculation. For trypan blue staining, TEM investigation, quantitative real-time RT-PCR, ABA measurement, and next-generation sequencing analysis, 4- to 5-week-old transgenic plants were sprayed with 20 μ M β -estradiol (Sigma-Aldrich, St. Louis) every 12 h for 48 h before harvest.

Constructions.

DNA fragments encoding full-length and N-terminal-truncated AvrXccC (XC_2004) were amplified from *X. campestris* pv. *campestris* 8004 genomic DNA by PCR using AccuPrime pfx DNA polymerase (Invitrogen, Carlsbad, CA, U.S.A.). For protoplast transient expression analysis, PCR products were subcloned into pWEN18 vector by restriction site reconstructions to generate C-terminal YFP-tagged constructs under the control of *Cauliflower mosaic virus* 35S promoter. For generation of transgenic plants, PCR products were subcloned into pER8 vector under the control of the *XVE* promoter (Zuo et al. 2000) by restriction site reconstructions. All plasmids were verified by DNA sequencing. Primer sequences are listed in Supplementary Table S6.

Arabidopsis protoplasts analysis.

To observe subcellular localizations of YFP-tagged effectors, *Arabidopsis* mesophyll protoplasts were prepared according to Yoo and associates (2007). Briefly, leaves from 4- to 5-week-old plants grown in a relatively short photoperiod were used. Cellulase R10 and Macerozyme R10 obtained from Yakult Pharmaceutical Ind. Co. were prepared for enzyme solution to digest leaves. Plasmid DNAs were purified by plasmid Maxi kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. After digestion and wash, fresh protoplasts were transfected with 10 μ g of plasmid DNAs using the polyethylene glycol-calcium method. After 12 h of incubation at room temperature, subcellular localizations of YFP-tagged effectors were observed by confocal laser-scanning microscopy and images were collected with the Olympus fluoview FV1000 system.

Arabidopsis transformations.

To obtain *Arabidopsis* transgenic plants expressing AvrXccC₈₀₀₄ (1 to 440 aa) and AvrXccC₈₀₀₄ (110 to 440 aa), plasmid DNAs were introduced into *A. tumefaciens* strain ABI or EHA105 by the freeze-thaw method (Weigel and Glazebrook 2006). *Agrobacterium*-mediated transformation of *Arabidopsis* Col-0 plants was performed using the floral-dip method (Clough and Bent 1998). After transformation, seed were collected and selected on half-strength Murashige and Skoog (1/2 $\times$ MS) medium containing hygromycin (12.5 μ g ml⁻¹) and carbencillin (100 μ g ml⁻¹). For morphological examination, homozygous seed were obtained and germinated on 1/2 $\times$ MS medium containing 20 μ M β -estradiol. For transcriptome analysis, homozygous seed were grown on soil before β -estradiol treatment.

Trypan blue staining.

For the cell death assay, leaves were detached and stained with trypan blue according to van Wees (2008). Briefly, leaves were boiled in trypan blue-lactophenol solution (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) for 1 min, followed by overnight incubation at room temperature. After staining, samples were destained with chloral hydrate (2.5 g ml⁻¹) until the background was clear. Images were collected with Leica ZM75 microscopy.

Northern blotting.

For Northern blotting analysis, transgenic seed were germinated on 1/2 $\times$ MS medium. Seedlings (2 to 3 weeks old) were

treated with DMSO or 20 μ M β -estradiol for 12 h. Total RNA was extracted using Trizol reagent (Invitrogen) according to the manufacturer's instructions. Total RNA (12 μ g) was fractionated on 1.2% (wt/vol) agarose gel and then transferred to Hybond-XL membrane (GE Bioscience, Piscataway, NJ, U.S.A.). To detect transcripts of a deletion series of *AvrXccC₈₀₀₄*, the coding region of the *AvrXccC₈₀₀₄* gene was amplified and labeled with [α -³²P]dCTP using the random prime labeling system (GE Bioscience). Hybridization was performed overnight at 65°C in hybridization buffer (0.3 M sodium phosphate [pH 7.0], 10 mM EDTA, 5% [wt/vol] sodium dodecyl sulfate [SDS], 10% [wt/vol] dextran sulfate, and salmon sperm DNA at 0.15 mg ml⁻¹), and then washed with 0.1 $\times$ SSC (1 $\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and 0.1% (wt/vol) SDS at 65°C. Signals were detected by autoradiography.

TEM.

For ultrastructural analysis, leaves were detached and cut into transverse sections less than 2 mm wide. Samples were fixed with 2.5% (wt/vol) glutaraldehyde in 0.1 M phosphate buffer overnight at 4°C and then post-fixed with 1% (wt/vol) OsO₄. After being dehydrated in a graded series of acetone, samples were embedded in Spurr's resin mixture and cut by glass knives on an ultramicrotome. Ultrathin sections (70 nm) were collected with uncoated copper grids (75-mesh square) and stained with uranyl acetate and lead citrate. Sections were examined with a JOEL JEM-1400 transmission electron microscope at an acceleration voltage of 100 KeV. Images were collected with a digital camera.

Next-generation sequencing.

For RNA-Seq analysis, total RNA was extracted using the RNeasy plant mini kit (Qiagen) according to the manufacturer's instructions. RNA quality was determined using 1.2% (wt/vol) formaldehyde gel electrophoresis and an Experion RNA analysis kit (Bio-Rad, Munich). For Experion RNA analysis, RNA samples with an RNA quality indicator number greater than 9.3 were presented at each sample. Next-generation sequencing was done using Illumina Hiseq 2000. Sequence reads were aligned using CLC-bio. Differentially expressed genes were identified by DESeq. Gene expression levels were normalized as RPKM. GO analysis was performed by Blast2GO.

qRT-PCR.

For RNA expression analysis, leaves were collected and total RNA was extracted using Trizol reagent (Invitrogen) according to the manufacturer's instructions. cDNAs were synthesized by reverse transcription using Superscript III first-strand synthesis supermix (Invitrogen). qRT-PCR was performed using the KAPA SYBR fast qPCR kit (Kapa Biosystems, Boston) and Illumina Eco real-time PCR system to validate the RNA-Seq results. Expression levels of selected genes were normalized to the expression of reference gene *EF1 α* . Experiments were repeated at least three times.

Antibody production.

A DNA fragment encoding *AvrXccC₈₀₀₄* (110 to 440 aa) was amplified by PCR using AccuPrime Pfx DNA polymerase (Invitrogen). The PCR product was subcloned into pET-SUMO (Invitrogen) and verified by DNA sequencing. Recombinant protein His₆-SUMO-AvrXccC₈₀₀₄ (110 to 440 aa) was produced in *Escherichia coli* BL21 (DE3) cells and purified using Ni²⁺-NTA resin (Qiagen) according to the manufacturer's instructions. After being cleaved with Ulp1 to remove the His₆-SUMO tag, recombinant protein *AvrXccC₈₀₀₄* (110 to 440 aa) was purified for antibody production in rabbit. Specific poly-

clonal antibodies against *AvrXccC₈₀₀₄* were obtained by affinity purification using polyvinylidene difluoride membrane as a coupling matrix.

Measurement of endogenous ABA levels.

The extraction and measurement of endogenous ABA were performed following the method described by Hsu and associates (2011). After β -estradiol treatment, aerial parts of transgenic plants were collected and frozen immediately in liquid nitrogen. The frozen samples were lyophilized and their dry weights were measured. To measure the endogenous ABA content, 0.1-g dried samples were extracted with solution containing 80% (vol/vol) methanol and 2% (vol/vol) glacial acetic acid in the dark at 4°C. After overnight extraction on a rotary shaker, samples were centrifuged and the clear supernatants were dried with a speedvac concentrator. After being resuspended in Tris-buffered saline buffer, the endogenous ABA levels were determined with a Phytodetek ABA enzyme immunoassay test kit (Agdia, Elkhart, IN, U.S.A.) according to the manufacturer's instructions. Briefly, reactions were incubated at 37°C for 2 h, and the absorbance values were read at 405 nm by an enzyme-linked immunosorbent assay reader. Experiments were repeated at least three times.

ABA treatment and bacterial inoculation.

ABA treatment was performed following the method described by de Torres-Zabala and associates (2007). *X. campestris* pv. *campestris* 8004 strains were cultured in NY broth (3 g of beef extract, 5 g of peptone, and 3 g of yeast extract in 1 liter of water) at 28°C. *Arabidopsis* Col-0 plants (4 to 5 weeks old) were used for bacterial growth assay. At 24 h after plants were sprayed with 100 μ M ABA (Acros, Waltham, MA, U.S.A.), leaves were infiltrated with a bacterial suspension (2 $\times$ 10⁶ CFU ml⁻¹) by a syringe. The inoculated plants were kept at 22°C in a growth chamber. Bacterial populations in leaves were determined at indicated time intervals using NY agar medium containing rifampicin (50 μ g ml⁻¹). Control assays were done by using plants sprayed with a solution of 0.2% (vol/vol) ethanol. Experiments were repeated at least three times.

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