The phytopathogen *Xanthomonas campestris* scavenges hydroxycinnamic acids *in planta* via the *hca* cluster to increase virulence on its host plant

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Abstract

*Xanthomonas campestris* pv. *campestris* (*Xcc*) is the causal agent of black rot of cruciferous plants, which harbor high levels of hydroxycinnamic acids (HCAs) in their above‑ground parts. Thus, upon infection of the host plant, the pathogen experiences a complex cocktail of HCAs. The present study shows that *Xcc* can efficiently degrade the HCAs, 4‑hydroxycinnamic acid (4‑HCA), ferulic acid (FA) and sinapic acid (SiA), via an *hca* cluster which encodes putative genes for a 4‑hydroxycinnamoyl‑CoA synthetase/4‑HCA ligase HcaL, a benzaldehyde dehydrogenase HcaD, a 4‑hydroxycinnamoyl‑CoA hydratase/lyase HcaH and a member of the MarR‑family of transcriptional factors, HcaR. *Xcc* also degrades the HCA caffeic acid, but with an alternative mechanism. RT‑PCR and subsequent GUS assays show that the *hca* cluster is transcribed within a single operon, and its transcription is specifically induced by 4‑HCA, FA and SiA. Furthermore, we show that HcaR negatively regulates *hca* transcription when its ligand, the proposed degradation pathway intermediate HCA‑CoA, is not present. HcaR specifically binds to a 25‑bp site, which encompasses the ‑10 elements of the *hca* promoter. Finally, GUS histochemical staining and subsequent quantitative analysis shows that the *hca* cluster is transcribed *in planta* during pathogenesis of Chinese radish, and *hca* deletion mutant strains exhibit compromised virulence in cabbage. Together, these results suggest that the ability to degrade HCAs contributes to *Xcc* virulence by facilitating its growth and spread, and by protecting the pathogen from HCA toxicity. A working model to explain *Xcc* HCA sensing and subsequent induction of the HCA degradation process is proposed.

Keywords: *Xanthomonas campestris*, 4‑Hydroxycinnamic acid, *hca* cluster, Host–pathogen interaction, Pathogenicity

Background

Plants produce diverse phenolic compounds, among which are the hydroxycinnamic acids (HCAs) such as 4‑hydroxycinnamic acid (4‑HCA), 2‑hydroxycinnamic acid (2‑HCA), ferulic acid (FA), sinapic acid (SiA) and caffeic acid (CA) (El‑Seedi et al. 2012). In *Brassica*, HCAs are abundant and often found conjugated to sugar moieties or other hydroxylic acids (Olsen et al. 2009; Lin and Harnly 2010; Cartea et al. 2010). In addition to their crucial roles in plant growth and development, HCAs also play important roles in plant defence. HCAs are broadly antimicrobial; they disrupt membrane integrity and decouple the respiratory proton gradient (Harris et al. 2010). HCAs also reinforce protective physical barriers in plants by cross‑linking primary cell wall polysaccharides and by serving as precursors for lignin, which is a natural phenolic polymer that adds strength and rigidity to the plant cell wall (Naoumkina et al. 2010; Campos et al. 2010).
Due to their antioxidant and antimicrobial activities, the increased accumulation of phenolic acids can aid in plant defence against pathogens. For example, grapevines infected with *Xylella fastidiosa* accumulated FA in their xylem cell walls (Wallis and Chen 2012), and 4-HCA and FA were induced in barley root exudates after *Fusarium* inoculation (Lanoue et al. 2011). The catabolism of HCAs has been reported in several environmental or rhizosphere bacterial species such as *Rhodopseudomonas palustris* CGA009, *Sphingobium* sp. SYK-6, *Agrobacterium fabrum*, *Pseudomonas*, *Acinetobacter* sp. ADP1, *Ralstonia solanacearum* and *Rhodococcus jostii* RHA1 (Parke and Ornston 2003; Lowe et al. 2015; Hirakawa et al. 2012; Campillo et al. 2014; Calero et al. 2018; Meyer et al. 2018). The two major pathways for the degradation of 4-HCA into 4-hydroxybenzoic acid (4-HBA) are the CoA-dependent β-oxidation and the CoA-dependent non-β-oxidation pathways (Parke and Ornston 2003; Campillo et al. 2014; Calero et al. 2018). Both pathways are initiated by CoA activation of the 4-HCA carboxylate side chain to generate a hydroyxcinnamoyl-CoA. In *Rhodopseudomonas palustris*, *Acinetobacter* sp. ADP1 and *Ralstonia solanacearum*, the catabolism of 4-HCA, FA and CA follows the CoA-dependent non-β-oxidation pathway (Parke and Ornston 2003; Hirakawa et al. 2012; Kasai et al. 2012).

*X. campestris* pv. *campestris* (*Xcc*) is an obligate aerobic, gram-negative bacterium that causes black rot, a devastating, systemic, vascular disease of *Brassica* species (Vincente and Holub 2013). Due to its agricultural importance, numerous studies on its diverse virulence factors have established *Xcc* as a model organism for the study of plant-pathogen interactions (Mansfield et al. 2012; Timilsina et al. 2020). As a vascular phytopathogen, *Xcc* is exposed to diverse phenolic compounds during infection of its host plant. In addition to the endogenously biosynthesized phenolic acids, multiple degradative enzymes produced by *Xcc* cleave the plant cell wall to generate simple sugars and also release lignin, which, when hydrolyzed forms various types of phenolic acids such as HCAs, vanillic acid and 4-HBA (Bertani et al. 2001). Our previous findings revealed that *Xcc* can sense 4-HBA and convert 4-HBA into protocatechuate (PCA) via the *pobA/pobR* locus (Chen et al. 2020). PCA can be further converted into succinyl-CoA and acetyl-CoA via the β-ketoacid pathway (Wang et al. 2015). Disruption of the pathogen’s 4-HBA or PCA degradation pathways results in significantly reduced virulence (Wang et al. 2015; Chen et al. 2020), suggesting that successful *Xcc* infection may be partially dependent on the catabolism of plant-derived phenolic compounds. How *Xcc* overcomes cell wall lignification and HCAs to cause disease is not yet fully understood.

In this current study we initially analyze the role of an *hca* gene cluster in 4-HCA, FA and CA catabolism, and characterize the expression patterns of this gene cluster in *Xcc* strain XC1. The molecular mechanisms underlying the inductive transcription of this gene cluster are further investigated, and finally we show that this gene cluster is transcribed during *Xcc* infection of Chinese radish and is required for full pathogenicity of *Xcc*.

**Results**

**Cabbage leaf tissues contain pico‑molar levels of HCAs**

To quantitatively determine the relative level of HCAs in cabbage leaf tissues, micro levels of commercially available 4-HCA, FA, SiA, CA, 2-HCA and 3-HCA in aqueous solution were assayed using Ultra-High Performance Liquid Chromatography coupled with Triple Quadrupole Tandem Mass Spectrometry (UHPLC-TQT-MS). Quantitative standard curves were established for each of the HCAs (Fig. 1a and Additional file 1: Fig. S1). Endogenous HCAs were then extracted from the leaves of 2-month-old cabbage plants using the previously described methods of Pan et al. (2010). UHPLC-TQT-MS analysis identified 4-HCA, FA, SiA and CA from these cabbage leaf tissues at levels ranging from 55.6 to 173.2 pmol/g fresh weight (Fig. 1b). We could detect neither 2-HCA nor 3-HCA in cabbage leaf tissues.

**Xcc degrades 4-HCA into 4-HBA via the hca gene cluster**

To determine whether *Xcc* can degrade HCAs, 4-HCA, FA, SiA, CA, 2-HCA and 3-HCA were independently added to separate NYG cultures of wild-type strain XC1 at a final concentration of 50 μM. The cultures were then collected every 12 h for quantitative assay of HCA levels. The levels of 4-HCA, FA, SiA and CA decreased over time during XC1 growth in NYG medium (Fig. 1c), whereas the levels of HCAs incubated in NYG medium without the addition of XC1 did not change significantly (Additional file 1: Fig. S2a). FA was degraded most efficiently by XC1, followed by 4-HCA, SiA and CA. 2-HCA and 3-HCA were not degraded by XC1 (Fig. 1c).

Previously, the gene cluster PP_3356–PP_3359 was experimentally shown to be responsible for 4-HCA degradation in *Pseudomonas putida* strain KT2440.
Using this gene cluster as a template and the Xcc strain B100 DNA sequence, Blastp analysis identified the homologous gene cluster XCCB100_3542–XCCB100_3545 (hca in this study) which encodes putative genes for 4-hydroxycinnamoyl-CoA synthetase/4-HCA ligase (HcaL), benzaldehyde dehydrogenase (HcaD, 490 aa), 4-hydroxycinnamoyl-CoA hydratase/lyase (HcaH) and a multiple antibiotic resistance regulator (MarR)-family transcriptional regulator (HcaR) (Fig. 2a). Although sequence analysis indicated that Xcc strains XC1, CN14 and CN16 contained this same hca gene cluster (Additional file 1: Fig. S3a; https://www.ncbi.nlm.nih.gov/genome/), the hca cluster in Xcc strains ATCC33913 and 8004 was comprised of 5 genes (Additional file 1: Fig. S3a). In these strains the loss of a G residue in the hcaD DNA sequence caused a frameshift which resulted in a stop codon and a truncated 384 aa HcaD (Additional file 1: Fig. S3b). Consequently, a new gene (Xcc0808 in ATCC33913 or XC_3422 in 8004) was annotated in these two strains downstream of hcaD (Additional file 1: Fig. S3b).

To examine the possible involvement of the Xcc hca gene cluster in 4-HCA catabolism, a series of mutant strains, ΔhcaL, ΔhcaD and ΔhcaH, were generated in the wild-type strain XC1. When grown in NYG medium supplemented with 50 µM 4-HCA, all the mutant strains displayed similar growth to the wild-type strain XC1 (Fig. 2b). The 4-HCA levels in the XC1 culture declined over time while the 4-HCA levels in the cultures of ΔhcaL and ΔhcaH remained relatively constant during growth (Fig. 2c). Overexpression of hcaL or hcaH via the vector pBBRMCS in ΔhcaL or ΔhcaH restored their 4-HCA degradation abilities to the level of wild-type XC1 (Fig. 2c). These results suggest that hcaL and hcaH are essential for 4-HCA degradation in XC1. In contrast, 4-HCA levels in the ΔhcaD culture declined over time, although they remained significantly higher than those in the XC1 culture at all growth points (Fig. 2c). Overexpression of hcaD in the mutant strain ΔhcaD restored the 4-HCA degradation ability to that of the wild-type strain XC1 (Fig. 2c). These results suggest that although HcaD is also involved in 4-HCA degradation, a HcaD homolog may be present in XC1.

Our previous results showed that Xcc can produce 4-HBA via xanB2 and degrade 4-HBA via pobA and the.

(Plaggenborg et al. 2003). Using this gene cluster as a template and the Xcc strain B100 DNA sequence, Blastp analysis identified the homologous gene cluster
Fig. 2 4-HCA degradation in strain XC1 of _Xcc_ via the _hca_ gene cluster. 

**a** Using the gene cluster PP_3356–PP_3359 which is involved in 4-HCA degradation in _Pseudomonas putida_, homology analysis identified a putative 4-HCA degradation gene cluster, _hca_ in _Xcc_ (colored arrows). _hcaL_ encodes a putative 4-hydroxycinnamoyl-CoA ligase; _hcaD_ encodes a benzaldehyde dehydrogenase; _hcaH_ encodes a 4-hydroxycinnamoyl-CoA hydratase/lyase, and _hcaR_ encodes a MarR-family transcriptional regulator. The numbers inside the parentheses indicate the amino acid identity.

**b** Growth time course of XC1 and XC1-derived mutants in NYG medium supplemented with 50 μM 4-HCA.

**c** Time course for 4-HCA production by strain XC1, XC1-derived mutants and restored mutants in NYG cultures supplemented with 50 μM 4-HCA. 

**d** A simple schematic diagram of 4-HBA synthesis, utilization and degradation processes in _Xcc_.

**e** Time course of 4-HCA and 4-HBA production of strains ΔxanB2ΔxanA2ΔpobA (ΔIII) and ΔxanB2ΔxanA2ΔpobAΔhcaL (ΔIIIΔhcaL). 

**f** The proposed 4-HCA degradation pathway in _Xcc_. For the data in (b, c, and e), three independent experiments were conducted for each and the averages with standard deviations are shown.
in this study) was generated. This triple mutant strain cannot produce 4-HBA and also cannot degrade 4-HBA; thus, it can be used to test whether 4-HBA is the end product of 4-HCA degradation via the hca gene cluster. When mutant strain ΔIII was grown in NYG medium supplemented with 50 μM 4-HCA, 4-HCA levels decreased over time while 4-HBA levels in the same culture increased over the same time period (Fig. 2e). Furthermore, when hcaL was deleted in strain ΔIII to generate strain ΔIIIΔhcaL, which was then grown in NYG medium supplemented with 50 μM 4-HCA, 4-HCA levels remained constant, and significant levels of 4-HBA were not detected (Fig. 2e). Taken together, these results strongly suggest that the end product of 4-HCA degradation via the hca cluster is 4-HBA (Fig. 2f).

Xcc also degrades FA and SiA, but not CA, via the hca gene cluster

Parent strain Xcc, and mutant strains ΔhcaL, ΔhcaL(hcaL), ΔhcaD, ΔhcaD(hcaD), ΔhcaH and ΔhcaH(hcaH) were individually grown in NYG medium supplemented with either 50 μM FA, 50 μM SiA or 50 μM CA, and the levels of these HCAs were examined during growth. Deletion of hcaL or hcaH abolished Xcc’s ability to degrade FA and SiA while deletion of hcaD significantly reduced the ability of Xcc to degrade FA and SiA (Additional file 1: Fig. S4a–d). Overexpression of either hcaL, hcaD or hcaH restored the mutants’ abilities to degrade FA and SiA (Additional file 1: Fig. S4a–d). In contrast, the deletion of hcaL, hcaD or hcaH had little effect on the ability of Xcc to degrade CA (Additional file 1: Fig. S2). These results suggest that Xcc uses the hca gene cluster to degrade FA and SiA but probably uses an alternative pathway to degrade CA. Based on previous findings and this current genetic study, the degradation pathways of FA and SiA in Xcc were proposed as shown in Additional file 1: Fig. S4e.

HcaR negatively regulates hcaR expression as well as the degradation of 4-HCA, FA and SiA

hcaR encodes a putative 156-amino acid transcriptional factor with a MarR-family helix-turn-helix (HTH) DNA binding domain (Fig. 3a). To test its effect on HCA degradation, hcaR was deleted in Xcc. The resultant strain ΔhcaR displayed a growth pattern similar to that of the wild-type strain Xcc in NYG medium supplemented with 50 μM 4-HCA (Fig. 3b). However, the 4-HCA degradation activity of strain ΔhcaR was significantly higher than that of the wild-type strain Xcc (Fig. 3c). Overexpression of hcaR via the expression vector pBBR-1-MCS in strain ΔhcaR significantly restored 4-HCA degradation activity to a level higher than that of the wild-type strain Xcc (Fig. 3c). Although not as dramatic, the effects of hcaR on FA and SiA degradation were similar to those observed with 4-HCA (Fig. 3d, e). These results suggest that HcaR negatively regulates the degradation of 4-HCA, FA and SiA in Xcc.

Subsequently, two reporter strains, Xcc::P_{hca}·gusA and ΔhcaR::P_{hca}·gusA, were generated to verify the negative regulation of hca expression by HcaR. In the absence of HCAs, hca expression was not observed in Xcc::P_{hca}·gusA, however a high level of expression was observed in ΔhcaR::P_{hca}·gusA (Fig. 3f). These results suggest that HcaR negatively regulates hcaR expression and subsequently the degradation of 4-HCA, FA and SiA in Xcc.

Transcription of the hca gene cluster is negatively regulated by HcaR and specifically induced by 4-HCA, FA and SiA

In the P. putida 4-HCA degradation gene cluster, the gene encoding HcaR (PP_3359) is divergently transcribed from the genes encoding HcaL, HcaH and HcaD (PP_3356–PP_3358) (Fig. 2a). However, hcaR is transcribed in the same orientation as the other hca genes in Xcc strains (Fig. 2a and Additional file 1: Fig. S3), possibly all from the same promoter. We initially used PCR and reverse transcription-PCR (RT-PCR) analysis to test whether the Xcc hca cluster was transcribed as a single operon. PCR analysis of Xcc genomic DNA confirmed that specific primers designed to amplify hca intergenic regions were functional (Fig. 4a, lanes 1). Then, using RT-PCR of Xcc total RNA harvested at 24 hpi (hours post-inoculation) in NYG medium supplemented with 50 μM 4-HCA, we identified the same three specific PCR products that correspond to the three intergenic regions of the hca cluster (Fig. 4a, lanes 3). Finally, simple PCR reactions with these same RNA preparations gave negative results.

(See figure on next page.)

Fig. 3 The effect of HcaR on HCA degradation. a The domain organization of HcaR in Xcc. b Growth time course of Xcc, ΔhcaR and ΔhcaR(hcaR) in NYG medium supplemented with 50 μM 4-HCA. c–e Time course of HCA production by Xcc, ΔhcaR and ΔhcaR(hcaR) in NYG liquid medium supplemented with 50 μM 4-HCA, 50 μM 4-FA or 50 μM CA. f Relative GUS activities of two reporter strains, Xcc::P_{hca}·gusA and ΔhcaR::P_{hca}·gusA when grown on NYG agar or liquid medium in the absence of HCAs. Averages with standard deviations of three independent experiments are shown.
Fig. 3 (See legend on previous page.)

a  

\[ \text{HcaR} \quad \text{HTH MARR} \]

b  

\begin{align*}
\text{Cell density (OD}_600) & \quad 0 \quad 12 \quad 24 \quad 36 \\
\text{Time after inoculation (h)} & \end{align*}

\begin{align*}
\text{XC1} & \quad \text{ΔhcaR} & \quad \text{ΔhcaR(hcaR)} \\
\end{align*}

c  

\begin{align*}
\text{4-HCA (mM)} & \quad 0 \quad 12 \quad 24 \quad 36 \\
\text{Time after inoculation (h)} & \end{align*}

\begin{align*}
\text{XC1} & \quad \text{ΔhcaR} & \quad \text{ΔhcaR(hcaR)} \\
\end{align*}

d  

\begin{align*}
\text{PA (mM)} & \quad 0 \quad 12 \quad 24 \quad 36 \quad 48 \\
\text{Time after inoculation (h)} & \end{align*}

\begin{align*}
\text{XC1} & \quad \text{ΔhcaR} & \quad \text{ΔhcaR(hcaR)} \\
\end{align*}

e  

\begin{align*}
\text{SIA (mM)} & \quad 0 \quad 12 \quad 24 \quad 36 \quad 48 \\
\text{Time after inoculation (h)} & \end{align*}

\begin{align*}
\text{XC1} & \quad \text{ΔhcaR} & \quad \text{ΔhcaR(hcaR)} \\
\end{align*}

f  

XC1::P\text{\_hca\_gusA}  \quad \text{ΔhcaR::P\text{\_hca\_gusA}}  \quad \text{XC1::gusA}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure3}
\caption{(See legend on previous page.)}
\end{figure}
confirming the absence of DNA contamination (Fig. 4a, lanes 2). These results showing that the hca intergenic region sequences are found on mRNA suggested that hcaR and the other hca genes are all part of the same transcript. To further confirm these findings, each of the upstream DNA fragments (~500 bp) of hcaR, hcaH, hcaD and hcaL, (P_hca, P_hcaH, P_hcaD, P_hcaL, respectively), was cloned, individually fused with gusA, and used to generate the strains XC1::P_hca-gusA, XC1::P_hcaH-gusA, XC1::P_hcaD-gusA and XC1::P_hcaL-gusA (Additional file 1: Fig. S5a, b). These strains were then used for promoter activity assays. When tested on either NYG agar or in NYG liquid medium supplemented with 50 mg/L X-gluc and 50 μM 4-HCA, only strain XC1::P_hca-gusA displayed GUS activity (Fig. 4b). These results confirmed that only P_hca has promoter activity and that hcaR is co-transcribed with the other hca genes as a single operon.

Using the constructed reporter strain XC1::P_hca-gusA, hca transcription was also monitored in the presence of other hydroxycinnamic acids. On NYG agar medium supplemented with 4-HCA, FA or SiA (50 μM and 200 μM), colonies were blue, indicating that hca expression was induced (Fig. 4c). In contrast, using agar plates supplemented with CA, 4-HBD or vanillin, no GUS-dependent blue color was observed in the reporter strain XC1::P_hca-gusA (Fig. 4c). During growth in liquid NYG culture, the P_hca-dependent GUS activities increased up to 50-fold as the concentrations of 4-HCA, FA or SiA increased from 50 to 200 μM (Fig. 4c). However, the addition of CA, 4-HBD or vanillin had little effect on the GUS activities of XC1::P_hca-lacZ (Fig. 4c). These results suggest that hca transcription is specifically induced by 4-HCA, FA and SiA.

4-HCA-CoA, FA-CoA and SiA-CoA are ligands of HcaR
The ligand-free form of MarR-family transcriptional factors binds to cognate nucleotide operator sequences to repress transcription, and this binding is antagonized in the presence of a ligand. For example, in the 4-HCA-degrading soil bacterium Rhodococcus jostii RHA1, the HcaR homolog CouR first forms a dimer and then binds two molecules of the catabolite 4-HCA-CoA. Thus, the presence of 4-HCA-CoA prevents the binding of CouR to its operator DNA in vitro (Otani et al. 2016). Based on the degradation pathways of 4-HCA, FA and SiA (Fig. 2f and Additional file 1: Fig. S4e), in the presence of 50 μM HCAs, HCA-CoAs should be accumulated in strain ΔhcaH, but not in strain ΔhcaL. To study the ligand of HcaR in Xcc, two reporter strains ΔhcaL::P_hca-gusA and ΔhcaH::P_hca-gusA were generated in this study. On NYG agar plates supplemented with 50 μM 4-HCA, FA or SiA, the two reporter strains grew well and P_hca-dependent GUS activity was observed in strain ΔhcaH::P_hca-gusA but not in strain ΔhcaL::P_hca-gusA (Fig. 5a). Similar results were observed when these strains were grown in liquid NYG medium supplemented with 50 μM HCAs (Fig. 5b). These results demonstrate that the HcaL-catalyzed reaction products, 4-hydroxycinnamoyl-CoA, feruloyl-CoA and sinapoyl-CoA are ligands of HcaR.

HcaR specifically binds to a 25-bp site in the hca promoter region
Heterologous expression of the full-length Xcc hcaR gene in E. coli via the expression vector pET-28a yielded soluble protein (Fig. 6a), and purified HcaR formed a dimer in solution (Additional file 1: Fig. S6). Electrophoretic mobility shift assays (EMSA) were then conducted to examine the DNA-binding activity of HcaR to a 183 bp DNA fragment encompassing the hca promoter region, P_hca (10 nM). As shown in Fig. 6b, a significant shift of labeled DNA was observed in the presence of increasing concentrations (0.05–5.0 nM) of HcaR. In the presence of high levels of unlabeled probes (2000 nM), no more shift was observed (Fig. 6b, lane 8). These results indicate a specific binding between HcaR and P_hca.

To identify the precise HcaR binding site, a DNase I footprinting analysis was performed. A representative sequencing result is shown in Fig. 6c. The data show that HcaR can protect a 25-bp DNA region “CTAGATTGT ATTTCATGGATAAAT” from DNase I digestion. The HcaR binding site fully overlaps the -10 box in the hca promoter (Fig. 6d). Additionally, an alignment of the hca promoter sequences from 9 Xanthomonas genomes revealed a putative inverted repeat sequence ATTGTA T–ATACAA (Additional file 1: Fig. S7). Such inverted repeats are characteristic of the nucleotide sequences...
Fig. 4 (See legend on previous page.)

**a**

Diagram showing the gene expression relationships between hcaL, hcaD, hcaH, and hcaK with arrows indicating regulatory interactions.

**b**

Bar graph showing the relative GUS activity for different constructs of the Xc1::gusA gene under the control of hcaL, hcaD, hcaH, and hcaA promoters in the presence of 50 μM 4-HCA.

**c**

Bar graph showing the GUS activity/OD500 for different compounds at 50 and 200 μM concentrations, including 4-HCA, FA, SiA, CA, 4-HBD, and vanillin, with significant differences indicated by asterisks.

Legend:
- Xc1::gusA
- Xc1::P_{hcaL-gusA}
- Xc1::P_{hcaD-gusA}
- Xc1::P_{hcaH-gusA}
- Xc1::P_{hcaA-gusA}
recognized by MarR family transcriptional regulators (Otani et al. 2016). When the ATT GTA T sequence was converted to CCC CCC C and the resultant variant was subjected to EMSA analysis for HcaR binding ability, this variant was unable to bind to HcaR (Fig. 6e). This result verifies the key role of the inverted repeat sequence in HcaR binding.

The hca cluster is transcribed during XC1 infection of Chinese radish

Xcc is a xylem-dwelling phytopathogen and the conditions for Xcc in planta growth are probably different from those required for growth in culture. To elucidate the role of the hca cluster in Xcc pathogenesis, it must be determined whether the hca cluster is expressed during disease development. To this end, the XC1 strain carrying the hca reporter gene construct XC1::P_hca-gusA was introduced into Chinese radish. XC1::gusA carrying a promoterless gusA gene and the previously constructed pobA reporter strain XC1::P_pobA-gusA (Chen et al. 2020) were used as negative and positive control strains, respectively.

Histochemical staining analysis detected P_hca-dependent GUS expression in the infected leaf tissues (Fig. 7a). No significant differences in bacterial population levels (CFUs) were observed between the reporter strains in any of the infected leaves analyzed at 3 and 5 days post-inoculation (dpi) (Fig. 7a). Subsequent GUS quantitative assays demonstrated that the expression level of the hca cluster was comparable to that of pobA inside the infected tissues (Fig. 7b).

The hca cluster is required for full virulence of XC1 in cabbage

We further characterized the virulence levels of hca mutants, ΔhcaL, ΔhcaD, ΔhcaH and ΔhcaR, in cabbage (jingfeng-1) using the leaf-clipping method (Fig. 7c). At 15 dpi, the average lesion lengths of strains ΔhcaL, ΔhcaD, ΔhcaH and ΔhcaR in cabbage were 8.3 mm, 10.2 mm, 3.9 mm and 11.2 mm, respectively (Fig. 7d); all significantly shorter than that of the wild-type strain XC1 (15.9 mm).

The hca cluster is conserved in the phytopathogens Xanthomonas

A BLAST analysis of the NCBI Nr database revealed that the hca cluster is present in the genomes of almost all Xanthomonas species, including: Xcc, X. citri pv. citri, X. campestris pv. raphanin, X. campestris pv. vesicatoria,
Fig. 6  (See legend on previous page.)
X. oryzae pv. oryzicola, X. oryzae pv. oryzae, X. fuscans subsp. fuscans, X. phaseoli pv. phaseoli and X. albilineans (Additional file 1: Fig. S8). These findings suggest that HCA degradation is a conserved mechanism in the phytopathogen Xanthomonas.

Discussion

4-HCA, FA, SiA and CA are commonly found in plants; therefore, phytopathogens may experience a complex cocktail of HCAs during infection of their eukaryotic host plants. Using Xcc as a model bacterial pathogen, this study for the first time provides a systematic insight into the role of the hca gene cluster in HCA degradation and Xcc virulence. We also describe a unique HcaR-dependent regulatory mechanism for hca transcription. Our results first show that cabbage produces pico-molar levels of 4-HCA, FA, SiA and CA; second, Xcc degrades 4-HCA, FA and SiA via the hca gene cluster; third, 4-HCA, FA and SiA specifically induce the expression of the hca gene cluster for the initiation of the differential degradation of these HCAs; fourth, HCA-derived HCA-CoAs are ligands of HcaR; fifth, HcaR negatively regulates hca transcription by binding to a 25-bp region within the promoter P_{hca}; sixth, the hca gene cluster is expressed by Xcc after infection of Chinese radish, at the onset of black rot symptom development; and finally, this gene cluster is required for the full virulence of Xcc XC1 on host plants. In light of these findings, it is likely that the degradation of HCAs via the hca gene cluster may simultaneously help the pathogen to survive against plant antimicrobial compounds, to spread by reducing physical barriers, and to multiply using HCAs as a carbon source. Given its importance in Xcc pathogenicity and its conservation in all Xanthomonas species, HCA degradation represents a new type of virulence factor which could be
targeted in the development of new strategies to prevent *Xanthomonas* pathogenesis.

A range of environmental strains and the two phytopathogens *R. solanacearum* and *A. fabrum* were reported to degrade HCA; however, the genes responsible for HCA degradation and the molecular mechanisms for *hca* induction have only been studied in a limited number of strains including *A. fabrum*, *Sphingobium* sp. SYK-6, *Acinetobacter* sp. ADP1, *Pseudomonas fluorescens* BF13 and *R. jostii* RHA1 (Parke and Ornston 2003; Calisti et al. 2008; Kasai et al. 2012; Otani et al. 2016). The precise mechanism of HCA induction of *hca* transcription remains incompletely characterized, although it is widely accepted that the transcription of *hca* genes is induced by HCAs and HcaR is a repressor of *hca* transcription. Consistent with these previous findings, this study showed that the expression of *hca* is specifically induced by 4-HCA, FA and SiA, but not by CA, 4-HBD or vanillin (Fig. 4), and the ligands of HcaR include 4-HCA-CoA, FA-CoA and SiA-CoA (Fig. 5).

In all the previously identified HCA degradation clusters, the regulatory gene encoding HcaR and the degradative enzyme-encoding genes are divergently transcribed, implying a trans-regulatory pattern (Otani et al. 2016; Kamimura et al. 2017; Meyer et al. 2018) (Fig. 2a). The present study shows that in all *Xanthomonas* sp, *hcaR* and all other *hca* genes are transcribed within the same operon (Fig. 2a and Additional file 1: Fig. S8). This cis-regulatory pattern does not seem sufficient on its own for the regulation of *hca* expression in *Xcc*. Thus, it is possible that an additional, as yet uncharacterized, trans-acting regulatory mechanism provides fine tuning of *hca* expression. Based on current knowledge, we propose a schematic working model for *hca* induction in *Xanthomonas* (Fig. 8). In the absence of HCAs or during the epiphytic stage of Xcc infection, the *hca* cluster is transcribed at a low level. HcaR binds to site 1 of the *hca* promoter P_{hca}, allowing a low level of *hca* transcription (Fig. 8a). In the presence of HCAs or during the *in planta* stage, these molecules bind to an uncharacterized sensor protein HcaX and the resultant HCA/HcaX complex binds to site 2 of P_{hca} to enhance *hca* transcription (Fig. 8b). The increased levels of HcaL catalyze the conversion of HCAs into HCA-CoAs, which further bind to HcaR. The HCA-CoA/HcaR complex then releases from the site 1 of P_{hca}, further increasing the transcription level of *hca*. This is a self-reinforcing process which eventually leads to very high levels of expression of the *hca* gene cluster.

Lowe et al. (2015) showed that HCA degradation protects *R. solanacearum* from plant chemical defenses and contributes to successful root colonization and virulence. The present study clearly demonstrates the expression of *hca* during Xcc infection of Chinese radish, suggesting that HCA degradation plays a role in Xcc pathogenesis. Furthermore, our study also shows that the deletion of individual genes from the *hca* gene cluster significantly reduces Xcc virulence in cabbage. Although mutant strains Δ*hcaL*, Δ*hcaH* and Δ*hcaD* were all significantly
reduced in virulence compared to strain XC1, ΔhcaD showed a significantly higher virulence than that of strains ΔhcaL and ΔhcaH (Fig. 7). This is not surprising since ΔhcaD retained considerably higher levels of HCA degradation than those of the other two mutant strains (Fig. 2 and Additional file 1: Fig. S4). In contrast, although strain ΔhcaR showed significantly greater degradation of HCAs than strain XC1 as expected, its virulence was significantly lower than that of XC1 (Fig. 7). This inconsistency could be due to the participation of HcaR in other Xcc metabolic pathways. Similar findings were also reported in A. fabrum C58, in which the MarR family transcriptional regulator HcaR not only negatively regulated HCA degradation, but also modified virB gene expression (Meyer et al. 2018). The A. fabrum hcaR deletion mutants showed a contrasting competitive colonization ability, being less abundant than the wild-type strain in tumors but more abundant in the rhizosphere (Meyer et al. 2018).

4-HCA, 4-HBA, SiA, vanillic, CA, and FA are all found at relatively high concentrations in wastewater effluent from the production of pulp and paper as well as olive oil, and they are also found in waste products from the crop straw utilization industry. The conventional physicochemical methods for degradation of these toxic compounds, include superior processes such as photocatalytic degradation and electrochemical oxidation (Gernjak et al. 2003; Elaoud et al. 2011). Alternatively, microbiological processes for the transformation and degradation of toxic organic pollutants are now promising methods for the remediation of industrial wastewater. Although various bacterial strains are able to metabolize 4-HCA, FA, 4-HBA and CA, few of them are effective with SiA (Xie et al. 2016). With this study, it is now clear that Xcc can transform four of these pollutants including SiA with known mechanisms, and the fifth (CA) via an unknown mechanism. Our findings provide a basis for the practical application of Xanthomonas in the bioremediation of HCA-contaminated industrial wastewater and soil, and in the synthetic biology for converting HCAs into value-added products.

Conclusions

Xcc efficiently degrades the HCAs 4-HCA, FA, and SiA via the hca gene cluster. The transcription of hca cluster is specifically induced by 4-HCA, FA, and SiA, but not by CA. HcaR specifically binds to a 25-bp site within the -10 elements of the hca promoter, and negatively regulates hca transcription. The hca cluster is transcribed in planta during pathogenesis of Chinese radish, and hca deletion mutant strains exhibit compromised virulence in cabbage. These results suggest that degradation of HCAs contributes to Xcc virulence by facilitating its growth and spread.

Methods

Bacterial strains and culture conditions

The bacterial strains and plasmids used in the present study are listed in Additional file 2: Tables S1 and S2. In most of our experiments, Xcc wild-type strain XC1 and its derivatives were grown at 28 °C in NYG medium (5.0 g/L peptone, 3.0 g/L yeast extract and 20.0 g/L glycerol), whereas E. coli strains were grown at 37 °C in LB medium (10 g/L tryptone, 5 g/L yeast extract, 20 g/L NaCl, pH 7.0). Antibiotics were added at the following concentrations when required: rifampicin (Rif) 25 μg/mL, kanamycin (Km) 50 μg/mL, gentamicin (Gm) 20 μg/mL and ampicillin (Amp) 100 μg/mL. Bacterial growth was determined by measuring optical density at a wavelength of 600 nm.

Gene deletion and functional complementation analysis

Xcc in-frame deletion mutants were generated using previously described methods (He et al. 2006); Briefly, the upstream and downstream regions of the target gene (~500 bp) were fused by overlap extension PCR using the primers listed in Additional file 2: Table S3. The fusion product was then subcloned into the suicide vector pK18mobsacB. The resultant recombinant plasmid was introduced into XC1 and integrated into the target DNA via homologous recombination. The resultant strain was then plated on LB agar plate with 50 μg/mL Rif and 5% (w/v) sucrose. PCR and subsequent DNA sequencing were used to verify the deletion mutants. For complementation analysis, the target gene was PCR amplified using the primers listed in Additional file 2: Table S3 and cloned into the multiple cloning site of the expression plasmid pBBR-1-MCS2. The resultant constructs were transferred into Xcc by triparental mating.

Extraction and quantitative analysis of HCA levels in cabbage leaf tissues

Cabbage (Jingfeng-1) was grown in a growth cabinet (NINBO YANGHUI, RND-400B) at 25 °C and 75% humidity with a photoperiod of 16 h (8000 Lx) for two months. HCAs were extracted from 200 mg of leaf tissues following previously developed methods (Pan et al. 2010). The dry extracts were then dissolved in 50 μL of methanol, and 5 μL extracts were loaded onto UHPLC-TQT-MS (Agilent, USA) for quantitative analysis, following the procedures described by Chen et al. (2020). Commercially available 4-HCA, FA, SiA and CA (Sigma, USA) were serially diluted to plot standard curves for quantification of endogenous HCA levels in leaf extracts (Additional file 1: Fig. S1).
**Extraction and quantitative analysis of HCAs in Xcc cultures by high performance liquid chromatography (HPLC)**

HCAs in *Xcc* cultures were extracted and quantified as described previously (Chen et al. 2020). Briefly, 0.5 mL of cell culture was collected at either 12, 24 or 36 hpi, the pH was adjusted to 3.5, and then 1 mL of ethyl acetate was added for the extraction. After evaporation, the dry extract was dissolved in 200 μL methanol for HPLC analysis. The HCA levels were quantified using the peak area for the HPLC eluate following the established formula.

**Construction of gusA-dependent reporter strains and GUS activity assay**

A ~500-bp DNA fragment upstream of the translation initiation codon of the *hca* gene cluster was amplified by PCR using the primer sets in Additional file 2: Table S3. The PCR product was subsequently cloned into the *BamHI* and *HindIII* sites of the plasmid pMD18T-T0T1-gusA (Zhao et al. 2014). DNA fusion fragments harboring the T0T1 terminator, the promoter region and gusA gene were amplified by PCR using the primer set T0T1-F and gusA-R (Additional file 2: Table S3). The fusion genes were then cloned into the *Smal* and *KpnI* sites of pmjni-Tn7T-Gm plasmid (Choi and Schweizer 2006) to generate the plasmid mini-*P*<sub>hca</sub>-gusA. The plasmid mini-*P*<sub>hca</sub>-gusA was then integrated into strain XC1 by electroporation as described previously (Choi and Schweizer 2006). GUS activity assays were performed following the protocol described by Chen et al. (2020).

**Protein expression and purification**

The *hcaR* coding region was amplified with the primers listed in Additional file 2: Table S3 and fused to the expression vector pET-28a (Merck KgaA, Darmstadt, Germany). The resultant vector was then transformed into the *E. coli* strain BL21(DE3). The expression of HcaR was induced by adding 0.1 mM IPTG to the culture at OD<sub>600</sub> of 0.6 and the culture was grown at 18 °C for 16 h. Purification of the recombinant protein was by Ni<sup>2+</sup>-affinity chromatography using His-Pur Ni–NTA Resin (Thermo Scientific, Pierce Biotechnology, USA) according to the manufacturer’s instructions. HcaR was eluted with buffer containing 20 mM sodium phosphate, 300 mM sodium chloride and 250 mM imidazole (pH 7.4).

**Electrophoretic mobility shift assay (EMSA)**

EMSA was performed according to the methods described previously (Chen et al. 2020). The 183-bp DNA probe corresponding to the sequence from -159 to 24 of the *hcaR* promoter region was amplified by PCR using the primer set cy5-<sub>P</sub>hca-F/R. Cy5-labeled probe (10 nM) was incubated with HcaR at a range of concentrations in EMSA buffer (20 mM Tris pH 7.9, 2 mM DTT, 10 mM MgCl<sub>2</sub>, 5% glycerol, 40 μg/mL BSA, 100 ng/mL sonicated salmon sperm DNA). To verify the specificity of the binding, 2000 nM unlabeled probe was amplified and added as a specific competitor. After incubation at 25 °C for 30 min, the reaction mixture was electrophoresed at 4 °C on a 4.5% native polyacrylamide gel in 0.5 × Tris-borate-EDTA (TBE) buffer for 45 min at 125 V. The gels were scanned for fluorescent DNA using the Starion FLA-9000 Scanner (FujiFilm, Japan).

**DNase I footprinting sequencing assay**

The fluorescent FAM-labeled probes were amplified by PCR with Dpx DNA polymerase using the primer set of M13F-47 (FAM) and M13R-48 and plasmid pUC19-T-<sub>P</sub>hca as a template. DNase I footprinting assays were conducted in TOLO Biotech, Shanghai following a protocol described previously (Wang et al. 2012).

**Plant GUS histochemical staining assay and bacterial CFU determination in planta**

*Xcc* strains were cultured in NYG liquid medium overnight, adjusted to an OD<sub>600</sub> of 0.1 (1.0 × 10<sup>8</sup> CFU/mL), and were then used to inoculate the leaves of Chinese radish (*Raphanus sativus* Manshenhong) using the leaf clipping method (Li et al. 2014). At 3 and 5 dpi, the infected leaves were collected for GUS histochemical staining and β-glucuronidase activity analysis following the procedure by Li et al. (2014). *Xcc* CFU in planta were also quantified in a parallel experiment. For each strain, three leaves were chosen for GUS staining and 5 leaves for CFU quantification in each experiment; the experiments were repeated twice. *Xcc* strains harboring the plasmids pLAFR3 or pGUShrpX (Li et al. 2014) were used as negative and positive controls, respectively.

**Virulence assay in cabbage**

The virulence of *Xcc* in cabbage (*Brassica oleracea*) cultivar Jingfeng-1 was tested by the leaf-clipping method (Chen et al. 2020). The third true leaf of 2-month-old cabbage plants were inoculated with sterile scissors dipped in bacterial suspensions (OD<sub>600</sub> value of 0.1). Lesion length was measured at 15 dpi. For each strain, a total of 15 plants were inoculated and the average lesion lengths with standard deviation are shown.

**Statistical analysis**

All experiments were performed at least three times unless otherwise stated. ANOVA for experimental data sets was performed using JMP software version 5.0 (SAS Institute Inc., Cary, NC). Significant effects of treatment were determined by the F value (*P* = 0.05). When a
significant F test was obtained, separation of means was accomplished by Fisher's protected LSD (least significant difference) test at $P < 0.05$.

**Abbreviations**

2-HCA: 2-Hydroxycinnamic acid; 3-HCA: 3-Hydroxycinnamic acid; 4-HBA: 4-Hydroxybenzoic acid; 4-HCA: 4-Hydroxycinnamic acid; 4-HCA-CoA: 4-Hydroxycinnamoyl-CoA; CA: Caffeic acid; CoA: Coenzyme A; FA: Ferulic acid; FA-CoA: Feruloyl CoA; HCA: Hydroxycinnamic acid; HMPHP-CoA: (3,5-Dimethoxy-4-hydroxyphenyl)-propionyl-CoA; HMPHP-CoA: (3-Methoxy-4-hydroxyphenyl)-propionyl-CoA; HTH: Helix-turn-helix; NMG medium: Peptone yeast glycerol medium; PCA: Protocatechuic acid; RT-PCR: Reverse transcription-PCR; SIA: Sinapic acid; SIA-CoA: Sinapoyl CoA; T3SS: Type III secretion system; TBE: Tris-borate-EDTA; UHPLC-TQ‑MS: Ultra‑High‑Performance Liquid Chromatography coupled with Triple Quadrupole Tandem Mass Spectrometry; Xcc: X. campestris; Pv: campesstri.

**Supplementary Information**

The online version contains supplementary material available at https://doi.org/10.1186/s42483-022-00119-z.

**Additional file 1. Fig. S1.** HPLC-Triple Quadrupole Mass Spectrometry analysis of 4-HCA and FA. a Parameters used for HPLC-Triple Quadrupole Mass analysis of 4-HCA and FA. b The mass spectrometry spectrum of 4-HCA and FA. c, d The plotted standard curve between peak area and the concentration of 4-HCA and FA. Fig. S2. CA degradation in strain XC1 and several hca gene cluster mutant strains. a Time course of HCA levels in NMG medium. b Growth time course of XC1 and mutant strains in NMG medium supplemented with 50 μM CA. c Time course of CA degradation in XC1 and XC1-derived strains grown in NMG medium supplemented with 50 μM CA. Three independent experiments were conducted and data are shown as averages with standard deviations. Fig. S3. Genomic analysis of the hca gene cluster in Xcc strains. a The hca gene cluster in Xcc strains b100, ATCC33913 and 8004. b The hcaD DNA sequence in ATCC33913 and 8004. c hcaD in Xcc strain XC1. Fig. S4. FA and SIA degradation by the hca gene cluster a, c Growth time course of XC1 and XC1-derived strains in NMG medium supplemented with 50 μM FA or SIA. b, d Time course of FA and SIA degradation by XC1 and XC1-derived strains in NMG liquid culture supplemented with 50 μM FA or SIA. e The proposed FA and SIA degradation pathways in Xcc. HMPHP-CoA, (3-methoxy-4-hydroxyphenyl)-propionyl-CoA; PCA, protocatechuic acid; HMPHP-CoA, (3, 5-dimethoxy-4-hydroxyphenyl)-propionyl-CoA. For a-d, three independent experiments were conducted for each and the averages with standard deviations are shown. Fig. S5. Construction of gusA transcriptional reporter strains. a The cloned putative promoter regions for detection of transcriptional activity. b Schematic representation of the promoter-gusA reporter strain. Fig. S6. The evidence for HcaC dimer formation. a The standard relationship between protein size and elution volume on a Superdex 200 column in Tris buffer (10 mM Tris, 150 mM NaCl, 5% glycerol, pH 7.5). b, c Size exclusion chromatography analysis of HcaC in Tris buffer. Fig. S7. Conservation analysis of the HCA binding site in Xanthomonas strains. Xcc: X. campestris pv. campesstri XC1; Xoc: X. oryzae pv. oryzae BLS256; Xccl: X. campesstri pv. campesstri; Xflf: X. fuscans subsp. fuscans 4834R; Xcv: X. campestris pv. vesicatoria 85–10; Xccl: X. campesstri pv. campesstri 306; Xcr: X. campesstri pv. raphani 756C; Xcc: X. campesstri pv. vesicatoria 85–10; Xcc: X. campesstri pv. campesstri 306; Xcr: X. campesstri pv. raphani 756C.

**Additional file 2. Table S1.** Bacterial strains used in this study. Table S2. Plasmids used in this study. Table S3. Oligonucleotide primers used in this study.

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**Author contributions**

YWH conceived the experimental design, interpreted the results and wrote the manuscript. ARP and LZ participated in the interpretation of the results and writing of the manuscript. BC, RFL and LZ performed the experiments. KS developed the assay method for promoter analysis. All authors read and approved the final manuscript.

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**Availability of data and materials**

Not applicable.

**Declarations**

**Ethics approval and consent to participate**

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**Consent for publication**

Not applicable.

**Competing interests**

The authors declare that they have no competing interests.

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