**Xanthomonas** transcriptome inside cauliflower hydathodes reveals bacterial virulence strategies and physiological adaptations at early infection stages

**ORIGINAL ARTICLE**

**Xanthomonas campestris pv. campestris** (Xcc) is a seed-transmitted vascular pathogen causing black rot disease on cultivated and wild Brassicaceae. Xcc enters the plant tissues preferentially via hydathodes, which are organs localized at leaf margins. To decipher both physiological and virulence strategies deployed by Xcc during early stages of infection, the transcriptomic profile of Xcc was analysed 3 days after entry into cauliflower hydathodes. Despite the absence of visible plant tissue alterations and despite a biotrophic lifestyle, 18% of Xcc genes were differentially expressed, including a striking repression of chemotaxis and motility functions. The Xcc full repertoire of virulence factors had not yet been activated but the expression of the HrpG regulon composed of 95 genes, including genes coding for the type III secretion machinery important for suppression of plant immunity, was induced. The expression of genes involved in metabolic adaptations such as catabolism of plant compounds, transport functions, sulphur and phosphate metabolism was upregulated while limited stress responses were observed 3 days postinfection. We confirmed experimentally that high-affinity phosphate transport is needed for bacterial fitness inside hydathodes. This analysis provides information about the nutritional and stress status of bacteria during the early biotrophic infection stages and helps to decipher the adaptive strategy of Xcc to the hydathode environment.

**KEYWORDS**

adaptation, **hrp** gene cluster, hrpG, hydathode, transcriptome, type III effector, type III secretion

---

**Abstract**

*Xanthomonas campestris pv. campestris* (Xcc) is a seed-transmitted vascular pathogen causing black rot disease on cultivated and wild Brassicaceae. Xcc enters the plant tissues preferentially via hydathodes, which are organs localized at leaf margins. To decipher both physiological and virulence strategies deployed by Xcc during early stages of infection, the transcriptomic profile of Xcc was analysed 3 days after entry into cauliflower hydathodes. Despite the absence of visible plant tissue alterations and despite a biotrophic lifestyle, 18% of Xcc genes were differentially expressed, including a striking repression of chemotaxis and motility functions. The Xcc full repertoire of virulence factors had not yet been activated but the expression of the HrpG regulon composed of 95 genes, including genes coding for the type III secretion machinery important for suppression of plant immunity, was induced. The expression of genes involved in metabolic adaptations such as catabolism of plant compounds, transport functions, sulphur and phosphate metabolism was upregulated while limited stress responses were observed 3 days postinfection. We confirmed experimentally that high-affinity phosphate transport is needed for bacterial fitness inside hydathodes. This analysis provides information about the nutritional and stress status of bacteria during the early biotrophic infection stages and helps to decipher the adaptive strategy of Xcc to the hydathode environment.
INTRODUCTION

*Xanthomonas campestris* pv. *campestris* (Xcc) is a seed-transmitted vascular pathogen causing black rot disease on Brassicaceae. This bacterium has a complex lifestyle composed of both epiphytic and endophytic stages (Vicente & Holub, 2013) that have been studied using molecular genetics since the 1980s (Daniels et al., 1984). Xcc epiphytic life is associated with environmental stresses such as UV light or dehydration and relies, for instance, on the production of xanthan exopolysaccharides (EPS) or protective pigments such as xanthomonadin. Upon favourable conditions, Xcc will gain access into the leaf inner tissues via wounds or hydathodes (Cerutti et al., 2017).

Hydathodes are plant organs mediating guttation localized at the leaf margin. Hydathodes are classically composed of an epidermis with water pores resembling stomata and an inner loose parenchyma called epithem irrigated by numerous xylem vessels (Cerutti et al., 2019). These specific structures offer an ecological niche for pathogenic bacteria and a rapid access to xylem vessels leading to systemic vascular infections (Cerutti et al., 2017). However, only a few pathogens have been demonstrated to colonize this niche and the conditions driving hydathode infection are poorly understood (Carlton et al., 1998; Cerutti et al., 2017; Fukui et al., 2000; Hugouvieux et al., 1998; Nino-Liu et al., 2006; Robene-Soustrade et al., 2006). While there is no evidence of a preinvasive immunity limiting Xcc entry through water pores, a postinvasive immunity was described inside the epithem (Cerutti et al., 2017). This is best revealed by the inability of a bacterial mutant of the hypersensitive response and pathogenicity (Hrp) type III secretion system to multiply in the epithem and to initiate vascular infections (Cerutti et al., 2017). The T3S system is responsible for the secretion and translocation of type III effector (T3E) proteins inside plant cells, where they interfere with plant physiology and suppress plant immunity (Buttner & Bonas, 2010). These results highlight the importance of immune suppression for the establishment of the infection.

Once inside hydathodes, Xcc adapts to this niche and adopts a biotrophic lifestyle: bacteria slowly multiply in the apoplastic spaces between epithem cells without causing visible tissue alterations as observed until 3 days postinfection (dpi) of cauliflower hydathodes (Cerutti et al., 2017). A switch to a necrotrophic behaviour is then observed, resulting in the almost complete digestion of the epithem at 6 dpi and Xcc vascularization. Systemic infection reaching the flowers will cause seed colonization and transmission to seedlings (Darsonval et al., 2009; Dutta et al., 2014).

During infection, Xcc may feed on guttation fluid, xylem sap or plant tissues (Duge de Bernonville et al., 2014). This process can be facilitated by high-affinity nutrient transport systems such as TonB-dependent transporters (TBDT) for mineral (e.g., iron) or carbohydrate nutrition (e.g., sucrose; Blanvillain et al., 2007) and a large repertoire of plant cell wall-degrading enzymes secreted through the type II secretion (T2S) system. Such metabolic adaptations need to be finely coordinated throughout the infectious cycle. Master regulators include the *rpf*-diffusible signal factor (DSF) quorum-sensing system, sensors of nutrient availability and metabolic activity and two-component systems. These regulation pathways allow bacterial cells to respond appropriately to diverse extracellular stimuli encountered during its life cycle, such as oxidative stress, oxygen levels, pH, temperature, and plant signals (He & Zhang, 2008; Qian et al., 2008; Tao et al., 2010). Among these regulators, some are well known to play a major role in virulence, such as the response regulator HrpG, which is, with the transcriptional regulator HrpX, the master regulator of the T3S regulon (Noël et al., 2001; Roux et al., 2015; Zhang et al., 2020).

Our knowledge of Xcc gene expression in planta remains elusive and technically challenging, especially at early stage of infection when bacterial populations are low. Indeed, most transcriptomic studies in Xcc or other *Xanthomonas* species have so far been performed in vitro focusing on specific regulons of the *hrpG* and *hrpX* genes in *hrp*-inducing media, of the *prc* protease gene, of the DSF-mediated quorum-sensing system, or of the *gum* genes responsible for xanthan production (Alkhateeb et al., 2016; An et al., 2014; Chen et al., 2018; Jalan et al., 2013; Kim et al., 2016; Li et al., 2019; Liao et al., 2016; Liu et al., 2013; Noh et al., 2016; Schmidtke et al., 2012; Zhang et al., 2013). Only a few in planta transcriptomic analyses have been performed on *Xanthomonas* (Getaz et al., 2020; Lee et al., 2017; Li et al., 2019; Liao et al., 2019) and all at late stages of infection and/or in comparison to in vitro-grown bacteria. Such in planta transcriptomic approaches would help identify new pathogenic behaviours and adaptation to the host during the infection process and in the different tissues colonized. Such approaches are also good descriptors of the environmental conditions and stresses imposed by the host on the bacterial pathogen (Nobori et al., 2018).

In this study, we compare the transcriptome of Xcc inside cauliflower hydathodes 4 or 72 hr after inoculation, that is, during the biotrophic stage of infection, to determine Xcc adaptive transcriptomic responses and infer the environmental conditions met by this bacterial pathogen inside hydathodes.

RESULTS

2.1 Improved annotation of Xcc strain 8004 genome based on a large transcriptomic data set

Transcriptomic analyses are intrinsically dependent on the proper structural annotation of genes. To improve annotation of the Xcc strain 8004 using experimental expression data, we produced the transcriptome of two nearly isogenic strains grown in MOKA medium: wildtype strain 8004 and strain 8004::*hrpG*+, which expresses the constitutively active variant E44K of the *hrp* response regulator *HrpG* (Guy et al., 2013b) (see later for comparative analysis of the *HrpG* regulon). Total RNAs were extracted from exponentially growing bacteria and subjected to ribodepletion as described (Roux et al., 2015). Small (<200 nucleotides [nt]) and large (>200 nt) RNA
fracti9ns were subjected to paired-end and single-end strand-specific sequencing, respectively. RNAs protected by a 5′ triphosphate group in the small RNA fraction were used to map precisely the transcriptional start sites. These 13 libraries corresponding to 88,130,360 and 135,564,422 reads from small and large RNA fractions, respectively, were used to refine genomic annotation. More than 1,724 transcriptional starts (5′ untranslated regions [UTRs]) and 1,246 3′ UTRs could be experimentally defined (Table 1, https://dx.doi.org/10.25794/reference/id52ofys) in the 3′ untranslated region. These results highlight the importance of experimentally supported genome annotations and offer improved resources for the functional analysis of Xcc transcriptome.

2.2 | Xcc transcriptome remodelling at early stages of hydathode infection

To capture a proxy of the physiological status of Xcc at the early stage of plant leaf infection, we performed RNA sequencing (RNA-Seq) on bacteria harvested from cauliflower hydathodes 72 hr after a rapid (15 s) dip inoculation of an attached leaf (Figure 1a). This 72 hr postinoculation (hpi) time point corresponds to a biotrophic phase of the infection where the bacteria are still limited to the epidermal apoplastic spaces (Cerutti et al., 2017). After 4 hr of infection, the bacteria coming from the initial inoculum. However, this timepoint is a relevant reference to investigate bacterial acclimation to the hydathode environment.

During hydathode infection (72 versus 4 hpi), Xcc massively reshaped its transcriptome with 828 differentially expressed genes (DEGs) corresponding to 18% of Xcc CDSs (Figure 1b, Tables S1 and S2). A Gene Ontology (GO) enrichment analysis identified 18 biological processes, such as catabolism, stress response and transport, that were significantly enriched in upregulated genes (Table 2). On the other hand, 13 GO terms were enriched among downregulated genes, with a strong overrepresentation of motility and chemotaxis categories (Table 2).

2.3 | Motility and chemotaxis of Xcc are strongly repressed processes inside hydathodes

Expression of most genes coding for biosynthesis of flagella and type IV pili and chemotaxis such as $\text{fliA}$ ($\text{XC}_2245$, 85-fold decrease) were strongly repressed at 72 hpi compared to 4 hpi. Motility and chemotaxis are key components of pathogenicity, especially for plant pathogens (Matilla & Krell, 2018). However, Xcc seems not to be flagellated when growing in xylem fluids and the motile Xcc cells seemed less pathogenic on cauliflower and radish (Kamoun & Kado, 1990). These observations suggest a probable fitness cost of motility during plant tissue colonization. To investigate the importance of motility in disease development, we constructed mutants in key genes for the synthesis of type IV pili ($\Delta \text{pilE}$ and $\Delta \text{pilA}$) or flagella ($\Delta \text{fliC}$ and $\Delta \text{fliQ}$). Single and multiple mutants were tested for in vitro motility (Figure S3a), pathogenicity (Figure S3b), and hydathode colonization (Figure 2c). Despite expected motility phenotypes in vitro (Figure S3a), none of the tested mutants affected disease symptom development or hydathode colonization (Figures S3b and 2c) as might have been expected for genes whose expression is repressed inside hydathodes. Altogether, these results suggest that motility is a process that is not needed for hydathode infection.
2.4 | Activation of the HrpG regulon at an early stage of hydathode infection

HrpG is a known master regulator required for the expression of the T3S machinery, T3E proteins, and additional genes including plant cell wall-degrading enzymes (PCWDEs) in *Xanthomonas* spp. (Noël et al., 2001; Roux et al., 2015). In Xcc, expression of *hrpG* and 49 (out of 55) genes coding for the T3S system and type III-secreted proteins was induced at 72 hpi, suggesting the involvement of HrpG at this stage of infection (Tables S1 and S3-3). We thus investigated the biological importance of *hrpG* during hydathode infection and studied its regulon.

The 8004 Δ*hrpG* mutant showed a 10-fold reduced multiplication in hydathodes at 72 hpi (Figure 2a) and was avirulent on cauliflower after wound inoculation (Figure S3b). Both phenotypes could be complemented (Figures 2a and S3b). These results are expected
because this mutant no longer expresses the T3SS, an essential virulence factor for Xcc.

To study the HrpG regulon independently of Xcc growth conditions, we decided to take advantage of a point mutation known to mimic HrpG phosphorylation, yielding a constitutively active form of HrpG named HrpG* (Wengelnik et al., 1999). This hrpG* (E44K) gain-of-function mutation conferring constitutive expression of the HrpG regulon in vitro did not affect the multiplication of Xcc in cauliflower hydathodes (Figure 2a) nor its pathogenicity after wound inoculation relative to the wildtype strain or EPS production (Figure S3b,c). These results suggest that the gain-of-function mutation carried by HrpG* and overexpression of T3SS (see below) do not affect virulence under the inoculation protocol used.

### Table 2

GO terms of biological processes enriched among Xanthomonas campestris pv. campestris genes differentially expressed in hydathodes (72 vs. 4 hr postinoculation)

| GO.ID      | Term                              | Annotated | Significant | Expected | Adjusted p values |
|------------|-----------------------------------|-----------|-------------|----------|-------------------|
| Upregulated                                      |           |            |            |          |                   |
| GO:0006073 | Cellular glucan metabolic process | 19        | 11          | 1.89     | $1.8 \times 10^{-8}$ |
| GO:0055114 | Oxidation-reduction process       | 399       | 74          | 39.78    | $8.9 \times 10^{-8}$   |
| GO:0070814 | Hydrogen sulphide biosynthetic process | 5       | 5           | 0.5      | $9.5 \times 10^{-6}$   |
| GO:0005992 | Trehalose biosynthetic process    | 5         | 5           | 0.5      | $9.5 \times 10^{-6}$   |
| GO:0006950 | Response to stress                | 123       | 25          | 12.26    | 0.00063            |
| GO:0019344 | Cysteine biosynthetic process     | 6         | 4           | 0.6      | 0.00123            |
| GO:0009251 | Glucan catabolic process          | 11        | 5           | 1.1      | 0.00263            |
| GO:0043649 | Dicarboxylic acid catabolic process | 7       | 4           | 0.7      | 0.00265            |
| GO:0044247 | Cellular polysaccharide catabolic process | 8     | 4           | 0.8      | 0.00361            |
| GO:0009306 | Protein secretion                 | 46        | 11          | 4.59     | 0.00435            |
| GO:0043623 | Cellular protein complex assembly | 18        | 6           | 1.79     | 0.00612            |
| GO:0006457 | Protein folding                   | 18        | 6           | 1.79     | 0.00612            |
| GO:0098661 | Inorganic anion transmembrane transport | 15     | 5           | 1.5      | 0.01230            |
| GO:0098869 | Cellular oxidant detoxification   | 30        | 7           | 2.99     | 0.02468            |
| GO:0044419 | Interspecies interaction between organisms | 12    | 4           | 1.2      | 0.02506            |
| GO:0005975 | Carbohydrate metabolic process    | 201       | 39          | 20.04    | 0.03559            |
| GO:0006817 | Phosphate ion transport           | 8         | 3           | 0.8      | 0.03753            |
| GO:0016311 | Dephosphorylation                 | 33        | 7           | 3.29     | 0.04006            |
| Downregulated                                    |           |            |            |          |                   |
| GO:0006935 | Chemotaxis                        | 44        | 36          | 3.12     | $<10^{-30}$        |
| GO:0097588 | Archaeal or bacterial-type flagellum-dependent cell motility | 23 | 23 | 1.63 | $9.4 \times 10^{-28}$ |
| GO:0007165 | Signal transduction               | 175       | 51          | 12.4     | $1.7 \times 10^{-22}$ |
| GO:0044781 | Bacterial-type flagellum organization | 16    | 16          | 1.13     | $2.2 \times 10^{-19}$ |
| GO:0030031 | Cell projection assembly          | 16        | 13          | 1.13     | $3.5 \times 10^{-13}$ |
| GO:0000160 | Phosphorelay signal transduction system | 133    | 23          | 9.43     | $3.5 \times 10^{-5}$  |
| GO:0000272 | Polysaccharide catabolic process  | 27        | 7           | 1.91     | 0.0021             |
| GO:0007155 | Cell adhesion                     | 5         | 3           | 0.35     | 0.0031             |
| GO:0048870 | Cell motility                     | 25        | 25          | 1.77     | 0.0039             |
| GO:0071554 | Cell wall organization or biogenesis | 40    | 6           | 2.83     | 0.0149             |
| GO:0006468 | Protein phosphorylation           | 79        | 11          | 5.6      | 0.0215             |
| GO:0015031 | Protein transport                 | 81        | 12          | 5.74     | 0.0295             |
| GO:0000302 | Response to reactive oxygen species | 11      | 3           | 0.78     | 0.0378             |

*Number of genes annotated for a given GO term.
*Number of differentially regulated genes among the genes annotated for a given GO term.
*Expected number of differentially regulated genes if no significant enrichment.
*Terms for which all annotated genes are differentially regulated are highlighted in bold.
To determine the extent of the HrpG regulon, we compared the transcriptomes of the 8004::hrpG* and wildtype strains grown in MOKA medium (Tables S1 and S2). Wildtype strain 8004 does not express hrp genes in MOKA in contrast to strain 8004::hrpG*. Analysis of the HrpG regulon identified 95 DEGs (log$_2$(fold change) ≥2 or ≤−2, false discovery rate (FDR) adjusted p value <0.05) (Table S1). Among the 85 genes with an increased expression in strain 8004::hrpG*, 43 possess a plant-inducible promoter box (PIP box) and are thus probably under hrpX control (Table S1). Thirty-nine of the 95 DEGs correspond to genes involved in T3SS and T3Es (in red and blue in Table S1, respectively) explaining the enrichment in the GO term “secretion” (GO:0,046,903) among genes upregulated in strain 8004::hrpG* (Table 3). Expression of only 18 out of the 30 genes encoding type III secreted proteins was increased in strain 8004::hrpG* compared

**Figure 2** Xanthomonas colonization of hydathodes. Bacterial multiplication of Xanthomonas campestris pv. campestris (Xcc) 8004 wildtype strain (WT), deletion mutants, and complemented strains in individual hydathodes 3 and 6 days after dip-inoculation (dpi) of the second true leaf of 4-week-old cauliflower plants. The box plot representations show the impact of mutations (a) in the T3SS hrpG regulator, (b) in the phosphate and sulphate transport genes, and (c) in the motility genes over Xcc multiplication into hydathodes. Each point of the plot represents the population extracted from one hydathode. At least eight hydathodes were sampled on one leaf per plant and three plants were used per experiment, though not all hydathodes were infected. Results from at least three independent experiments were pooled and a total of at least 50 infected hydathodes were counted for each strain. Letters indicate statistically different groups obtained from the Kruskal–Wallis test on all data points for each strain with an error of $\alpha = 0.05$
to 24 genes at 72 hpi inside hydathodes (Table S3-3 and Figure S2c). Among the other 56 DEGs of the HrpG regulon, 26 encode proteins with unknown function and 19 PCWDEs. Altogether, these results illustrate that the HrpG regulon is not limited to the T3SS and T3Es, and that a part of Xcc's effectome expression regulation could rely on a HrpG/HrpX-independent pathway in planta.

The HrpG regulon was almost entirely included in the in planta transcriptome (90 out of 95 genes), indicating that HrpG is activated during hydathode infection (Figures 1b,c and S2b). Eighteen genes of the HrpG regulon were overexpressed at 4 hpi in comparison to the MOKA condition, indicating that expression of the T3S machinery is initiated early during infection.

### Table 3

| GO.ID          | Term                                      | Annotated | Significant | Expected   | Adjusted p values |
|----------------|--------------------------------------------|-----------|-------------|------------|-------------------|
| **BIOLOGICAL PROCESS** |                             |   |         |           |      |
| Upregulated    |                                             |   |         |           |      |
| GO:0002790     | Peptide secretion                          | 46 | 9         | 0.91       | $1.5 \times 10^{-7}$ |
| GO:0009306     | Protein secretion                          | 46 | 9         | 0.91       | $1.5 \times 10^{-7}$ |
| GO:0032940     | Secretion by cell                          | 47 | 9         | 0.93       | $1.8 \times 10^{-7}$ |
| GO:0046903     | Secretion                                 | 47 | 9         | 0.93       | $1.8 \times 10^{-7}$ |
| GO:0015031     | Protein transport                          | 81 | 10        | 1.61       | $2.5 \times 10^{-4}$ |
| GO:0008104     | Protein localization                       | 85 | 10        | 1.69       | $3.9 \times 10^{-4}$ |
| GO:0015833     | Peptide transport                          | 85 | 10        | 1.69       | $3.9 \times 10^{-4}$ |
| GO:0045184     | Establishment of protein localization      | 85 | 10        | 1.69       | $3.9 \times 10^{-4}$ |
| GO:0042886     | Amide transport                            | 86 | 10        | 1.71       | $4.3 \times 10^{-4}$ |
| GO:0033036     | Macromolecule localization                 | 96 | 10        | 1.91       | $1.2 \times 10^{-3}$ |
| GO:0071705     | Nitrogen compound transport                | 120 | 10        | 2.38       | $8.6 \times 10^{-3}$ |
| GO:0071702     | Organic substance transport                | 142 | 10        | 2.82       | 0.00035 |
| GO:0044419     | Interspecies interaction between organisms  | 12  | 3         | 0.24       | 0.00143 |
| GO:0051704     | Multiorganism process                      | 21  | 3         | 0.42       | 0.00763 |
| Downregulated  |                                             |   |         |           |      |
| GO:0000272     | Polysaccharide catabolic process            | 27 | 3         | 0.08       | $5.6 \times 10^{-5}$ |
| GO:0009057     | Macromolecule catabolic process             | 58 | 3         | 0.18       | 0.00057 |
| GO:0016052     | Carbohydrate catabolic process              | 61 | 3         | 0.19       | 0.00066 |
| GO:0005976     | Polysaccharide metabolic process            | 64 | 3         | 0.2        | 0.00076 |
| GO:0005975     | Carbohydrate metabolic process              | 201 | 4         | 0.63       | 0.00198 |
| **MOLECULAR FUNCTION** |                             |   |         |           |      |
| Upregulated    |                                             |   |         |           |      |
| GO:0016301     | Kinase activity                            | 176 | 8        | 2.71       | 0.0046 |
| GO:0001067     | Regulatory region nucleic acid binding     | 27 | 3         | 0.42       | 0.0077 |
| GO:0044212     | Transcription regulatory region DNA binding | 27 | 3         | 0.42       | 0.0077 |
| GO:0016798     | Hydrolase activity, acting on glycosyl bonds | 83 | 5         | 1.28       | 0.0081 |
| Downregulated  |                                             |   |         |           |      |
| GO:0004175     | Endopeptidase activity                      | 57 | 3         | 0.17       | 0.00045 |
| GO:0004252     | Serine-type endopeptidase activity          | 30 | 2         | 0.09       | 0.00315 |
| GO:0070011     | Peptidase activity, acting on L-amino acid peptides | 128 | 3   | 0.38       | 0.00477 |
| GO:0008233     | Peptidase activity                          | 152 | 3       | 0.45       | 0.00774 |
| GO:0016829     | Lyase activity                             | 158 | 3       | 0.46       | 0.00863 |
| GO:0008236     | Serine-type peptidase activity              | 53  | 2        | 0.16       | 0.00964 |
| GO:0017171     | Serine hydrolase activity                   | 53  | 2        | 0.16       | 0.00964 |

\(^a\) Number of genes annotated for a given GO term.

\(^b\) Number of differentially regulated genes among the genes annotated for a given GO term.

\(^c\) Expected number of differentially regulated genes if no significant enrichment.
(Table S1). However, the HrpG regulon (95 genes) remains a small part of Xcc transcriptomic adaptations to in planta conditions (828 genes).

### 2.5 Metabolic adaptations of Xcc inside hydathodes highlight several nutritional properties of the plant environment

Among the 31 GO terms differentially enriched at the transcriptomic level, a third are involved in metabolism, indicating that Xcc undergoes an important metabolic adaptation inside the hydathode.

#### 2.5.1 Modification of the expression profile of genes encoding PCWDEs

We observed an increased transcription of genes involved in the catabolism of cellulose, a major component of the primary cell wall, yet there was no increased expression of other PCWDE genes, nor of xps and xcs genes encoding type II secretion systems, at 72 hpi. Among the 43 genes encoding PCWDEs in Xcc strain 8004 (da Silva et al., 2002), 10 had an increased and 10 had a reduced expression at 72 hpi (Table S3-2). These observations are consistent with the absence of visible degradation of cell walls in the epithem and suggestive of a biotrophic lifestyle (Cerutti et al., 2017).

Lignin is another major component of plant cell walls and a source of aromatic compounds. Interestingly, the expression of XC_3426 and XC_3427 genes coding for protocatechuate (PCA) 4,5-dioxygenase subunits was increased 6- and 8-fold at 72 hpi, respectively. PCA is a lignin degradation product (Brown et al., 2004) that can be further catabolized by PCA dioxygenases to enter the tricarboxylic acid (TCA) cycle (Wang et al., 2015). While XC_3426 and XC_3427 relevance for pathogenicity remains unknown, XC_0375 to XC_0383 genes cluster encoding 3- and 4-hydroxybenzoate degrading enzymes are needed for full virulence of Xcc in radish (Wang et al., 2015), suggesting that degradation of plant phenolic compounds happens and matters inside plant tissues.

#### 2.5.2 Expression of transporter genes is remodelled in planta

Broad expression changes can be observed in transporter genes as 49 out of 210 genes involved in transport were differentially expressed at 72 hpi (Tables 2 and S3-1). Those transporters belong to the major facilitator superfamily (MFS), ATP binding cassette (ABC), and TonB-dependent transporter (TBDT) families. TBDTs have been shown to be involved in iron and carbohydrate polymer uptake with high affinity in Xcc (Blanvillain et al., 2007; Boulanger et al., 2010; Dejean et al., 2013). Fourteen out of 72 TBDT genes were differentially expressed: seven showed an increased expression at 72 hpi while seven were repressed. While most have no known function, those for which expression is induced by polygalacturonate (PGA) were less expressed at 72 hpi (Table S3-1) (Blanvillain et al., 2007). The expression of two TBDT genes, XC_3205 and XC_2512, which are both known to be positively regulated by HrpG and HrpX (Blanvillain et al., 2007), was induced at 72 hpi.

Interestingly, the absence of the fur regulon and its iron high affinity TBDT transporters (XC_0167, XC_3463, XC_2846, XC_1341, XC_1108, XC_0924-0925, XC_0642, XC_4249, XC_0558, and XC_4249) in our data set suggests that iron might not be limiting at this stage of hydathode infection.

#### 2.5.3 Upregulation of two pathways important for sulphur assimilation in hydathodes

In contrast to iron, induction of genes important for sulphur transport and assimilation was observed at 72 hpi in hydathodes, that is, the operon encoding the ABC sulphate transporter CysPUWA (XC_3292 to XC_3295) and the operon encoding an assimilatory sulphate reduction pathway leading to sulphide production (XC_0990 to XC_0994) were induced by 6- and 20-fold, respectively (Table S1). Sulphide is then available for cysteine and methionine biosynthesis. Sulphur metabolism including sulphur-containing amino acids, sulphur compounds, or sulphate has been shown to be involved in production of different virulence factors, as in xanthan production (Garcia-Ochoa et al., 2000) or T3SS induction (Schulte & Bonas, 1992). Yet, a deletion of the entire cysPUWA operon (ΔXC_3292-95) in Xcc strain 8004 did not significantly affect bacterial multiplication in hydathodes after dip-inoculation or disease symptom development after wound inoculation in cauliflower (Figures 2b and S3b). Thus, sulphate import through the CysPUWA system is not limiting for bacterial growth in hydathodes or sulphur might as well be acquired through other import pathways such as the taurine import system. For example, *Escherichia coli* responds to sulphate or cysteine starvation by expressing the ssuABCDE and tauABCD operons, which are involved in the uptake of alkanesulphonate and desulphonation of the organosulphonates and, uptake and desulphonation of taurine, respectively (Eichhorn et al., 2000; van der Ploeg et al., 1999). While ssuABCDE is absent in Xcc strain 8004, TauABCD homologues are encoded by genes of the locus XC_3454 to XC_3460. Interestingly, expression of these genes was increased at 72 hpi, suggesting a possible implication of this pathway in sulphur assimilation in planta.

#### 2.5.4 Phosphate uptake machinery is rate limiting for Xcc multiplication inside hydathodes

After 72 hr in hydathodes, the increased expression of the genes encoding the PstSCAB high-affinity transporter system (genes XC_2708 to XC_2711, 2- to 6-fold inductions; Tables S1 and S2), involved in active inorganic phosphate (Pi) import on phosphate starvation, suggests that Pi might be limiting inside hydathodes. This system is known to be activated in various conditions in bacteria (Lamarche et al., 2008),
including during plant colonization, and is essential for *Xanthomonas axonopodis pv. citri* (Xac) pathogenicity on citrus (Moreira et al., 2015; Pegos et al., 2014). To test if phosphate acquisition is important for Xcc strain 8004 during hydathode colonization, we mutated XC_2711 (pstB) and XC_3272, which encode a protein homologue of the PhoB response regulator important for the *E. coli* Pi starvation response (Gardner & McCleary, 2019; Yang et al., 2012). The results obtained demonstrated that pstB, unlike phoB, is important for hydathode colonization (Figure 2b) and that both ∆pstB and ∆phoB mutants caused symptoms similar to the wildtype strain after direct inoculation into xylem vessels (Figure S3b). Observation of both increased expression of the high-affinity Pi transport system and reduced multiplication of the high-affinity phosphate transporter mutant demonstrate that Pi is limiting during hydathode infection.

Similar to Pi, expression of genes important for nitrogen assimilation such as those involved in uptake of nitrite and nitrate and their reduction to ammonia (XC_2175 to XC_2178) were induced 3- to 9-fold at 72 hpi, further stressing the importance of Xcc mineral nutritional needs in hydathodes.

### 2.6 Xcc adapts to nutritional, osmotic, and environmental stresses in hydathodes

Surprisingly, there were limited transcriptional changes for genes associated with transcription, translation, replication, TCA cycle, or amino acid biosynthesis between 4 and 72 hpi. However, these functions are already strongly repressed at 4 hpi compared to MOKA conditions, indicating that adaptation to the plant environment is associated with a rapid repression of cellular division and core metabolism (Tables S1 and S2). Other stress-responsive genes had an increased expression in planta such as base excision and nucleotide excision repair systems, trehalose production pathways, superoxide dismutases, and chaperone proteins. In addition, expression of the ABC transporter system OpuB-ABC (Hoffmann et al., 2018, XC_0173 to XC_0174) and the choline degradation pathway (Wargo, 2013, XC_0760 to XC_0761), both involved in osmoprotection, were induced at 72 hpi, suggesting that bacteria face an osmotic shock in the apoplast of the epithem cells (Table S1). Finally, expression of some genes of the gum operon (XC_1658 to XC_1673) implicated in the production of the xanthan EPS were induced by 4- to 5-fold at 72 hpi. Xanthan is a well-known protectant against environmental stresses and toxic compounds, and prevents bacterium detection by plants (Kakkar et al., 2015). Altogether, these data seem to indicate that Xcc cells must cope with some nutritional, osmotic, and environmental stresses in hydathodes.

### 3 DISCUSSION

The Xcc life cycle depends on its adaptation to various plant environments (e.g., seeds, leaf surface, hydathodes, xylem, mesophyll, debris). This work describes the transcriptional changes that Xcc undergoes on plant infection and hydathode colonization, including the regulation of various metabolic and virulence pathways (Figure 3), and informs us about the environmental conditions faced by Xcc inside hydathodes.

#### 3.1 Xcc adopts a sedentary biotrophic lifestyle inside hydathodes

Xcc is traditionally reported to develop necrotic lesions associated with black rot disease (Vicente & Holub, 2013). However, the
physiological snapshot obtained by RNA-Seq at 72 hpi in hydathodes suggests that Xcc first behaves as a biotroph because the epithem is intact (Cerutti et al., 2017) and many genes coding for degradative enzymes, T2SS, and most catabolic pathways of sugar polymers and carbohydrates were expressed at low levels. We also observed that the constitutive activation of the HrpG regulon recapitulating part of the in planta condition at 72 hpi was associated with reduced extracellular protease activity (Figure S3d), as observed in *Xanthomonas euvesicatoria* (Noël et al., 2001). Xcc carbon and nitrogen needs could be supported in planta by the continuous flow of xylem sap in the epithem because xylem sap contains significant concentrations of carbohydrates, amino acids, and minerals, which can be metabolized at high affinity by Xcc (Duge de Beronville et al., 2014). Furthermore, the expression of multiple virulence-associated genes, such as those involved in quorum sensing, iron uptake, or motility, was also reduced at 72 hpi in hydathodes. Though motility has been shown to be an essential virulence trait for many bacterial pathogens (Chaban et al., 2015), Xcc motility mutants were not affected in pathogenicity as expected from genes whose expression is repressed in hydathodes. Repression of chemotaxis and motility has also been reported at early rice infection stages in *Xanthomonas oryzae pv. oryzicola* (Xoc) (Liao et al., 2019) while twitching motility and quorum sensing were both activated at later infection stages and are important for virulence of *Xanthomonas oryzae pv. oryzae* (Xoo) (Lee et al., 2017) and Xac (Li et al., 2019). Interestingly, an increase in the expression of genes important for twitching motility and adhesion was observed in Xcc grown in vitro in xylem sap (Duge de Beronville et al., 2014). Xylem sap corresponds to the environment met by Xcc immediately after leaving the hydathode. These observations suggest a possible biphasic infectious process with the reactivation of the motility and other virulence-associated genes at later infection stages. Further transcriptomic analyses of virulence gene expression throughout the entire infection cycle would be needed to support such a hemibiotrophic lifestyle of Xcc.

### 3.2 Stealthiness of Xcc inside hydathodes and neutralization of plant immune responses

The observed repression of chemotaxis, twitching, and swimming motility also suggests that those functions are dispensable if not detrimental once inside hydathodes. For instance, production of bacterial peptide flg22 from the flagellar FlIC protein is a well-known pathogen-associated molecular pattern (PAMP) recognized by the FLS2 receptor and a potent elicitor of basal plant immunity (Gomez-Gomez & Boller, 2000). While flg22, a peptide not recognized by *Arabidopsis* FLS2 (Guy et al., 2013a; Sun et al., 2006), we cannot exclude that other FlIC peptides, flagellar proteins, or pili proteins from Xcc strain 8004 could act as PAMPs in Brassicaceae. Xcc stealthiness could also be acquired by limiting bacterial multiplication until an efficient suppression of immunity has been achieved. In contrast to a wildtype Xcc strain, a T3S system mutant unable to deliver T3E proteins inside plant cells caused hydathode browning and necrosis at 48 hpi and had a reduced multiplication at 72 hpi (Cerutti et al., 2017). These results indicate that hydathode immune responses can be effective against bacterial pathogens and that their suppression by the Xcc T3S system and its T3E proteins is required for successful infection.

### 3.3 Inference of environmental conditions inside hydathodes based on Xcc transcriptomic behaviour

Xcc transcriptomic profiles can also be used to infer its metabolic and physiological status inside hydathodes and the nutritional properties of the epithem. For instance, the epithem environment is probably not limiting for assimilable iron because the corresponding uptake machinery is not expressed. In contrast, both the low-affinity phosphate inorganic transport (Pit) and the high-affinity phosphate-specific transport (Pst) systems important for the uptake of inorganic phosphate (Pi) in *Xanthomonas* are upregulated at 72 hpi (Moreira et al., 2015; Willsky & Malamy, 1980). In Xcc, we have observed that the expression of the Pst system is induced after 72 hpi in hydathodes. Although the Pst system is under the control of the PhoB response regulator, *phoB* was not differentially expressed in planta at 72 hpi in agreement with PhoB regulatory activity being dependent on its phosphorylation status rather than expression level (Gardner & McCleary, 2019). Similar to Xac (Moreira et al., 2015), the Xcc Pst system is needed for growth in planta. These results demonstrate that Xcc not only faces Pi starvation inside hydathodes but that Pi availability also limits Xcc proliferation in this tissue. Very low Pi concentrations are indeed found in the guttation fluids of several plant species, such as barley, (Nagai et al., 2013) and are correlated with expression of genes coding for plant high-affinity Pi transporters such as *AtPHT1;4* in hydathodes even under Pi-sufficient conditions (Misson et al., 2004). These observations suggest that an active competition between the plant and Xcc for access to Pi occurs in the epithem. Similar to Pi, Xcc transcriptome at 72 hpi also suggests that sulphur and nitrogen are present in low amounts, requiring the upregulation of dedicated uptake systems. Yet, it remains unclear whether these elements are limiting for growth of Xcc inside hydathodes. Exposure to stresses is also unveiled by the transcriptomic upregulation of genes involved in responses to general stress (e.g., gum genes) and osmotic stress. However, it remains uncertain whether osmotic stress is intrinsic of the epithemal environment or whether it is caused by plant immunity. For instance, we could not find significant evidence of oxidative stresses classically associated with strong plant immune responses. Therefore, Xcc seems to adapt rapidly to the low concentrations of nutrients found in the epithem and...
to endure limited stress, maybe due to the continuous flow of fluids inside hydathodes that renews nutrient supplies and dilutes potential antibacterial compounds.

Such a global gene expression study provides a picture of the bacterial population in planta and will feed future functional genomic approaches to Xcc pathogenicity.

4 | EXPERIMENTAL PROCEDURES

4.1 | Bacterial strains, plasmids, and growth conditions

The list of strains and plasmids used in this study is provided in Table S4. Xcc was cultivated in MOKA medium (4 g/L yeast extract, 8 g/L casamino acids, 1 mM MgSO$_4$, 2 g/L K$_2$HPO$_4$) at 28 °C under agitation at 200 rpm or on MOKA-agar plates (Blanvillain et al., 2007). E. coli TG1 and strain-carrying pRK2073 helper plasmid were cultivated in liquid Luria Bertani (LB) medium under agitation or on LB agar plates at 37 °C. Antibiotics were used at the following concentrations: 50 μg/ml rifampicin, 50 μg/ml kanamycin, and 40 μg/ml spectinomycin.

4.2 | Mutagenesis and complementation

In-frame deletion mutants of Xcc were obtained by double recombination with derivatives of the suicide plasmid pK18mobSacB as described (Schafer et al., 1994). Sequences flanking the deletion were amplified from Xcc strain 8004 genomic DNA and introduced into pK18mobSacB by Gibson assembly (Gibson et al., 2009). For complementation, the CDS was amplified from Xcc strain 8004 genomic DNA and cloned by Gibson assembly into plasmid pK18_CompR3, a pK18mobSacB derivative containing a pTac promoter and a T7 terminator region from PCZ1016 (Dejean et al., 2013) flanked by XC_1301 and XC_1302 sequences to drive stable insertion at the XC_1301/XC_1302 interval. For genes lacking a ribosome-binding site (RBS) in their upstream region, the RBS from plasmid pK18-GUS-GFP (Cerutti et al., 2017) was inserted downstream of the pTac promoter, giving pK18_compR3_RBS. All plasmids were conjugated into Xcc 8004::GUS-GFP derivatives by triparental mating with the E. coli TG1 carrying pRK2073 helper plasmid as described (Cerutti et al., 2017; Ditta et al., 1980; Figurski & Helinski, 1979). The sequences of oligonucleotides used to construct deletion and complementation plasmids are listed in Table S5. The growth of all strains was assessed in MME and MOKA media (Figure S4).

4.3 | Plant growth conditions

Brassica oleracea var. botrytis ‘Clovis F1’ (cauliflower) was grown under greenhouse conditions. Four-week-old plants were transferred 1 day before inoculations in a growth chamber (9 hr light, 22 °C, 70% relative humidity).

4.4 | Preparation of biological samples used for RNA-Seq

For in vitro RNA-Seq experiments, RNAs were extracted from Xcc strains grown in MOKA medium to exponential phase (OD$_{600}$ nm between 0.5 and 0.7), harvested by filtration as described (Roux et al., 2015), and stored at −80 °C.

For in planta RNA-Seq experiments, RNA was extracted from infected hydathodes sampled from the first three cauliflower leaves after inoculation by continuous or transient dipping in a bacterial suspension of 10$^8$ cfu/ml as described (Cerutti et al., 2017) using 0.01% of SILWET-L77 (DE SANGOSSE) (Figure 1a). These first three leaves of 4-week-old plants show mature hydathodes on leaf margins that can be successfully infected with Xcc. For inoculations by continuous dipping, detached leaves were dipped in the bacterial suspension for 4 hr and hydathodes were immediately harvested. For inoculations by transient dipping, leaves (attached to the plant) were dipped in a bacterial suspension for c. 15 s. Plants were placed in a domed propagator, watered, and covered for a day and a half (95%–100% humidity). Hydathodes were harvested 72 hpi. To determine the level of infection of hydathodes under both continuous and transient dipping inoculation protocols, the bacterial populations were determined at 4 and 72 hpi in 24 and 30 individual hydathodes, respectively (Figure S1b), as described below. Based on those results, these two inoculation methods allow the comparison of similar bacterial populations (around 10$^3$ bacteria per hydathode) having spent either 4 or 72 hr inside hydathodes.

To collect hydathodes, leaves were briefly rinsed twice in sterile distilled water and dried on a paper towel prior to harvesting. At least 1,000 hydathodes were macerated per condition with a 1.5 mm diameter punch. Collected tissues were immediately placed in RNA Protect Bacteria Reagent (Qiagen, 2:1 vol/vol) of RNA Protect and RNase-free water). After 3 min sonication in a water bath, the supernatant was recovered and centrifuged for 10 min at 5,000 × g. The pellet was stored at −80 °C. Three independent biological replicates were sampled.

4.5 | RNA extractions, ribodepletion, and sequencing

RNA extraction and ribodepletion were performed as previously described (Sallet et al., 2013). Oligonucleotide probes used for RNA depletion were directed against Xanthomonas rRNA and 2 rRNA (Ile and Ala) (Roux et al., 2015) and for plant-derived samples probes targeting Arabidopsis and Brassica oleracea rRNA and major chloroplastic RNA (Sallet et al., 2013) (Table S5). RNAs were fractionated into short (<200 nt) and long (>200 nt) RNA fractions using Zymo Research RNA Clean & Concentrator TM-5 columns (Proteigene) and subjected to oriented sequencing (see Supplementary material and methods for detailed procedures). Raw sequence data were submitted to the Sequence Read Archive (SRA) database (accessions SRP280320 and SRP280329).
**TABLE 4** Properties of RNAseq libraries from Xcc strain 8004 wild-type and derivatives grown in vitro or harvested from cauliflower hydathodes

| RNA size                  | Strain     | Condition                     | Biological replicates | Number of raw reads | Uniquely mapped reads | Hits _Brassica_ genes (%) | Hits _Xcc_ genes (%) | Accession       |
|---------------------------|------------|-------------------------------|-----------------------|----------------------|------------------------|--------------------------|----------------------|-----------------|
| Long RNAs fraction        | 8004       | MOKA                          | 3                     | 22 503 680           | 93.58                  | -                        | 9,472               | SRR12603737     |
|                           |            |                               |                       | 31 781 601           | 88.67                  | -                        | 9,433               | SRR12603738, SRR12603725 |
|                           |            |                               |                       | 29 317 932           | 88.54                  | -                        | 9,421               | SRR12603739, SRR12603726 |
| Small RNAs fraction       | 3          |                               |                       | 14 141 422           | -                      | -                        | -                   | SRR12603734     |
|                           | 13         |                               |                       | 13 443 960           | -                      | -                        | -                   | SRR12603735     |
|                           | 13         |                               |                       | 13 423 070           | -                      | -                        | -                   | SRR12603736     |
| Long RNAs fraction        | 8004::hrpG* | MOKA                          | 3                     | 21 746 450           | 9,587                  | -                        | 9,358               | SRR12603727     |
|                           |            |                               |                       | 14 282 665           | 94.84                  | -                        | 9,411               | SRR12603728     |
|                           |            |                               |                       | 15 932 094           | 95.08                  | -                        | 9,457               | SRR12603729     |
| Small RNAs fraction       | 4          |                               |                       | 10 314 675           | -                      | -                        | -                   | SRR12603720     |
|                           | 11         |                               |                       | 11 898 842           | -                      | -                        | -                   | SRR12603721     |
|                           | 10         |                               |                       | 10 794 476           | -                      | -                        | -                   | SRR12603722     |
|                           | 14         |                               |                       | 14 113 815           | -                      | -                        | -                   | SRR12603733     |
| 8004 cauliflower hydathodes 4 hpi | 3          |                               |                       | 158 422 507          | 33.42                  | 6,245                    | 1,332               | SRR12603813, SRR12603814, SRR12603815 |
|                           |            |                               |                       | 150 271 178          | 23.49                  | 6,621                    | 1,063               | SRR12603810, SRR12603811, SRR12603812 |
|                           |            |                               |                       | 173 591 985          | 41.15                  | 6,794                    | 1,012               | SRR12603804, SRR12603805, SRR12603806 |
| 8004 cauliflower hydathodes 72 hpi | 3          |                               |                       | 164 579 058          | 38.59                  | 5,519                    | 1,640               | SRR12603816, SRR12603817, SRR12603818 |
|                           |            |                               |                       | 163 894 181          | 38.47                  | 5,256                    | 2,727               | SRR12603801, SRR12603802, SRR12603803 |
|                           |            |                               |                       | 143 337 188          | 27.63                  | 73.48                    | 1.61                | SRR12603807, SRR12603808, SRR12603809 |

*a* Percentage of raw reads mapped uniquely to the sequences of Xcc strain 8004 chromosome (Genbank accession number CP000050.1), _Brassica oleracea_ nuclear and mitochondrial genomes (Accession GCA_000695525.1, NC_016118.1), and _Brassica rapa_ chloroplastic genome (BRARA_CHL, accession NC_040849.1).

*b* Percentage of unique hits to the genes of _Brassica oleracea_ nuclear and mitochondrial genomes and _Brassica rapa_ chloroplastic genome.

*c* Percentage of unique hits to the genes of Xcc strain 8004.

*d* Sequence Read Archive accession number.
4.6 | Reannotation of Xcc strain 8004 genome sequence

Annotation of *X. campestris* pv. *campestris* strain 8004 genome was performed using EuGene-PP (Sallet et al., 2013) (EuGene-PP v. 1.0, eugene-4.1c) with SRP280320 RNA libraries and *X. campestris* pv. *campestris* strains 8004, ATCC33913, and B100 public annotations GCA_000012105.1, GCA_000007145.1, and GCA_000070605.1, respectively (Qian et al 2005; Vorholter et al., 2008). This new annotation is available at https://dx.doi.org/10.25794/reference/id52ofys.

4.7 | Analysis of RNA-Seq results and statistical analysis

Mapping of RNA-Seq reads was performed on the Xcc strain 8004 reannotated genome sequence (Qian et al., 2005, Genbank accession number CP000050.1) and when appropriate on sequences of *B. oleracea* nuclear genome (Brassica_oleracea v. 2.1.31; accession GCA_000695252.1), *B. oleracea* mitochondrial genome (accession NC_016118.1), and *Brassica rapa* chloroplastic genome (BRARA_CHL, accession NC_040849.1) as described (Roux et al., 2015).

DEGs were detected with EdgeR Bioconductor package v. 3.30.3 (Robinson & Smyth, 2008). Genes with no counts across all libraries were discarded. Normalization was performed using the trimmed mean of M-values method (Robinson & Oshlack, 2010). Quality control plots of normalized data sets and reproducibility of biological repeats were generated by principal component analysis using the Ade4 v. 7-15 package (Dray & Dufour, 2007) and heatmaps obtained with the package pheatmap v. 1.0.12 (Raivo Kolde (2015), pheatmap: Pretty Heatmaps. R package v. 1.0.8. https://CRAN.R-project.org/package=pheatmap on sample-to-sample Euclidean distances.

Fitted generalized linear models (GLMs) with a design matrix multiple factor (biological repetition and factor of interest) were designed. The Cox–Reid profile-adjusted likelihood (CR) method in estimating dispersions was used. DEGs were called using the GLM likelihood ratio test using a false discovery rate (FDR) (Benjamini & Yekutieli, 2001) adjusted p value < 0.05. Clustering on filtered DEG (p < 0.05 in at least one biological condition) was generated with heatmap.2 function as available in the gplots Bioconductor package v. 3.0.1. (Warnes et al., 2015) using Ward’s minimum variance clustering method on Euclidean (Murtagh & Legendre, 2014). Analysis of GO enrichment was conducted using the topGO package v. 2.40.0 (Alexa et al., 2006).

4.8 | Infection of hydathodes and measurement of bacterial population

For hydathode infection, the second true leaf of cauliflower plants was dip-inoculated in a bacterial suspension at $10^8$ cfu/ml in 1 mM MgCl$_2$ containing 0.5% (vol/vol) Tween 80. To determine Xcc populations in single hydathodes, hydathodes were collected at 3 or 6 dpi by macrodissection with a 1.5 mm diameter punch.

Eight hydathodes were sampled per leaf and individually placed in 200 µl of 1 mM MgCl$_2$. After bead-assisted grinding at 30 Hz for 2 min using a Retsch MM400 grinder, 5 µl droplets of serial dilutions were spotted on MOKA plates supplemented with 30 µg/ml pimaricin in three technical replicates and incubated at 28 °C for 2–3 days. Individual colonies were counted and the mean of the three technical replicates was calculated to estimate the infection level of each hydathode. Experiments were performed on three plants per condition and in three independent biological replicates.

Significance of differences observed in bacterial population quantifications and bacterial pathogenicity assays were assessed using the nonparametric Kruskal–Wallis test with $\alpha = 0.05$.

ACKNOWLEDGEMENTS

A.C. and J.L. were funded by a PhD grant from the French Ministry of Higher Education, Research and Innovation. B.R., E.L., A.C., M.A., and L.D.N. were funded by grants from the Agence Nationale de la Recherche XANTHOMIX (ANR-2010-GENM-013-02), Xopaque (ANR-10-JCJC-1703-01), and NEPHRON (ANR-18-CE20-0020-01). We are grateful to Stéphanie Bolot for management and early analysis of the Xanthomix data set. J.L., E.L., A.B., R.B., M.A., and L.D.N. were funded by the XBOX (ANR-19-CE20-JCJC-0014-01) project. The Laboratoire des Interactions Plantes-Microbes-Environnement is part of the French Laboratory of Excellence project (TULIP ANR-10-LABX-41; ANR-11-IDEX-0002-02). All authors benefited from the COST action CA16107 EuroXanth.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

OPEN RESEARCH BADGES

This article has earned an Open Data badge for making publicly available the digitally-shareable data necessary to reproduce the reported results. The data is available at https://dx.doi.org/10.25794/reference/id52ofys.

DATA AVAILABILITY STATEMENT

Genome annotation accession numbers and sequence read archive accession numbers for RNA-Seq libraries are openly available as indexed in Tables 1 and 4, respectively.

ORCID

Julien S. Luneau https://orcid.org/0000-0002-1917-3468
Alice Boulanger https://orcid.org/0000-0001-5918-9547
Laurent D. Noël https://orcid.org/0000-0002-0110-1423

REFERENCES

Alexa, A., Rahnenfuhrer, J. & Lengauer, T. (2006) Improved scoring of functional groups from gene expression data by decorrelating GO graph structure. Bioinformatics, 22, 1600–1607.
Alkhatteeb, R.S., Vorhölter, F.-J., Rückert, C., Mentz, A., Wibberg, D., Hublik, G. et al. (2016) Genome wide transcription start sites analysis
Pegos, V.R., Nascimento, J.F., Sobreira, T.J., Pauletti, B.A., Paes-Leme, A., Qian, W., Han, Z.J. & He, C. (2008) Two-component signal transduction.

Noh, T.H., Song, E.S., Kim, H.I., Kang, M.H. & Park, Y.J. (2016) Transcriptome-based identification of differently expressed genes from Xanthomonas axonopodis pv. oryzae under in planta growth and in vitro culture conditions. The Plant Pathology Journal, 33, 458–466. 

Liao, C.-T., Liu, Y.-F., Chiang, Y.-C., Lo, H.-H., Du, S.-C., Hsu, P.-C. et al. (2016) Functional characterization and transcriptome analysis reveal multiple roles for Prc in the pathogenicity of the black rot pathogen Xanthomonas campestris pv. campestris. Research in Microbiology, 167, 299–312.

Liao, Z.X., Ni, Z., Wei, X.L., Chen, L., Li, Y.J., Yu, Y.H. et al. (2019) Dual RNA-seq of Xanthomonas oryzae pv. oryzae infecting rice reveals novel insights into bacterial-plant interaction. PLoS One, 14, e0215039.

Liu, W., Yu, Y.H., Cao, S.Y., Niu, X.N., Jiang, W., Liu, G.F. et al. (2013) Transcriptome profiling of Xanthomonas campestris pv. campestris grown in minimal medium MMX and rich medium NYG. Research in Microbiology, 164, 466–479.

Matilla, M.A. & Krell, T. (2018) The effect of bacterial chemotaxis on host infection and pathogenicity. FEMS Microbiology Reviews, 42, fux052.

Missen, J., Thibaud, M.C., Bechtold, N., Raghothama, K. & Nussaume, L. (2004) Transcriptional regulation and functional properties of Arabidopsis Pht1;4, a high affinity transporter contributing greatly to phosphate uptake in phosphate deprived plants. Plant Molecular Biology, 55, 727–741.

Moreira, L.M., Facincani, A.P., Ferreira, C.B., Ferreira, R.M., Ferro, M.I., Gozzo, F.C. et al. (2015) Chemotactic signal transduction and phosphate metabolism as adaptive strategies during citrus canker induction by Xanthomonas citri. Functional & Integrative Genomics, 15, 197–210.

Murtagh, F. & Legendre, P. (2014) Ward’s hierarchical agglomerative clustering method: which algorithms implement Ward’s criterion? Journal of Classification, 31, 274–295.

Nagai, M., Ohashi, M., Uehara, T., Yamagami, M., Miura, E., Kamakura, M. et al. (2013) Ion gradients in xylem exudate and guttation fluid related to tissue ion levels along primary leaves of barley. Plant, Cell and Environment, 36, 1826–1837.

Nino-Liu, D.O., Ronald, P.C. & Bogdanove, A.J. (2006) Xanthomonas oryzae pathovars: model pathogens of a model crop. Molecular Plant Pathology, 7, 303–324.

Nobori, T., Velasquez, A.C., Wu, J., Kvitko, B.H., Kremer, J.M., Wang, Y. et al. (2018) Transcriptome landscape of a bacterial pathogen under plant immunity. Proceedings of the National Academy of Sciences of the United States of America, 115, E3055–E3064.

Noël, L., Thieme, F., Nennstiel, D. & Bonas, U. (2001) cDNA-AFLP analysis unravels a genome-wide hrpG-regulon in the plant pathogen Xanthomonas campestris pv. vesicatoria. Molecular Microbiology, 41, 1271–1281.

Noh, T.H., Song, E.S., Kim, H.I., Kang, M.H. & Park, Y.J. (2016) Transcriptome-based identification of differently expressed genes from Xanthomonas oryzae pv. oryzae strains exhibiting different virulence in rice varieties. International Journal of Molecular Sciences, 17, 259.

Pegos, V.R., Nascimento, J.F., Sobreira, T.J., Pauletti, B.A., Paes-Leme, A. & Balan, A. (2014) Phosphate regulated proteins of Xanthomonas citri subsp. citri: a proteomic approach. Journal of Proteomics, 108, 78–88.

Qian, W., Han, Z.J. & He, C. (2008) Two-component signal transduction systems of Xanthomonas spp.: a lesson from genomics. Molecular Plant-Microbe Interactions, 21, 151–161.

Qian, W., Jia, Y., Ren, S.X., He, Y.Q., Feng, J.X., Lu, L.F. et al. (2005) Comparative and functional genomic analyses of the pathogenicity of phytopathogen Xanthomonas campestris pv. campestris. Genome Research, 15, 757–767.

Robene-Soustrade, I., Laurent, P., Gagnevin, L., Jouen, E. & Pruvost, O. (2006) Specific detection of Xanthomonas axonopodis pv. dieffenbachiae in anthurium (Anthurium andraeanum) tissues by nested PCR. Applied and Environmental Microbiology, 72, 1072–1078.

Robinson, M.D. & Oshlack, A. (2010) A scaling normalization method for differential expression analysis of RNA-seq data. Genome Biology, 11, R25.

Robinson, M.D. & Smyth, G.K. (2008) Small-sample estimation of negative binomial dispersion, with applications to SAGE data. Biostatistics, 9, 321–332.

Roux, B., Bolot, S., Guy, E., Denance, N., Lautier, M., Jardinaud, M.F. et al. (2015) Genomics and transcriptomics of Xanthomonas campestris species challenge the concept of core type III effector. BMC Genomics, 16, 975.

Sallet, E., Roux, B., Sauviac, L., Jardinaud, M.-F., Carrere, S., Faraut, T. et al. (2013) Next-generation annotation of prokaryotic genomes with EuGene-P: application to Sinorhizobium meliloti 2011. DNA Research, 20, 339–354.

Schafer, A., Tauch, A., Jager, W., Kalinowski, J., Thierbach, G. & Puhler, A. (1994) Small mobilizable multi-purpose cloning vectors derived from the Escherichia coli plasmids pK18 and pK19: selection of defined deletions in the chromosome of Corynebacterium glutamicum. Gene, 145, 69–73.

Schmidtke, C., Findeiss, S., Sharma, C.M., Kuhfuss, J., Hoffmann, S., Vogel, J. et al. (2012) Genome-wide transcriptome analysis of the plant pathogen Xanthomonas identifies sRNAs with putative virulence functions. Nucleic Acids Research, 40, 2020–2031.

Schulte, R. & Bonas, U. (1992) A Xanthomonas pathogenicity locus is induced by sucrose and sulfur-containing amino acids. The Plant Cell, 4, 79–86.

da Silva, A.C., Ferro, J.A., Reinaich, F.C., Farah, C.S., Furlan, L.R., Quaggio, R.B. et al. (2002) Comparison of the genomes of two Xanthomonas pathogens with differing host specificities. Nature, 417, 459–463.

Sun, W., Dunning, F.M., Pfund, C., Weingarten, R. & Bent, A.F. (2006) Specific detection of Xanthomonas oryzae pv. oryzae infecting rice re- gulator Clp defines a new class of cyclic di-GMP effectors. Journal of Bacteriology, 192, 1020–1029.

van der Plouw, J.R., Ivankowska, R., Bykowski, T., Hrynewicz, M.M. & Leisinger, T. (1999) The Escherichia coli ssuEDCB gene cluster is required for the utilization of sulfur from aliphatic sulfonates and is regulated by the transcriptional activator Cbl. Journal of Biological Chemistry, 274, 29358–29365.

Vicente, J.G. & Holub, E.B. (2013) Xanthomonas campestris pv. campestris (cause of black rot of crucifers) in the genomic era is still a worldwide threat to brassica crops. Molecular Plant Pathology, 14, 2–18.

Vorhölter, F.J., Schneiker, S., Goessmann, A., Krause, L., Békel, T., Kaiser, O., et al. (2008) The genome of Xanthomonas campestris pv. campestris B100 and its use for the reconstruction of metabolic pathways involved in xanthan biosynthesis. Journal of Biotechnology, 134, 33–45.

Wang, Y.J., Zhou, L., Chen, B., Sun, S., Zhang, W., Li, M. et al. (2015) A functional 4-hydroxybenzoate degradation pathway in the phytopathogen Xanthomonas campestris is required for full pathogenicity. Scientific Reports, 5, 18456.

Wargo, M.J. (2013) Homeostasis and catabolism of choline and glycine betaine: lessons from Pseudomonas aeruginosa. Applied and Environmental Microbiology, 79, 2112–2120.

Warnes, G., Bolker, B., Bonebakker, L., Gentleman, R., Huber, W., Liaw, A. et al. (2015) gplots: Various R programming tools for plotting data. R package version 3.0.1 Available at: https://CRAN.Rproject.org/package=gplots

Wengelnik, K., Rossier, O. & Bonas, U. (1999) Mutations in the regulator Clp defines a new class of cyclic di-GMP effectors. Functional genomics of Bacteriology, 192, 1020–1029.

Warnes, G., Bolker, B., Bonebakker, L., Gentleman, R., Huber, W., Liaw, A. et al. (2015) gplots: Various R programming tools for plotting data. R package version 3.0.1 Available at: https://CRAN.Rproject.org/package=gplots

Wengelnik, K., Rossier, O. & Bonas, U. (1999) Mutations in the regulator Clp defines a new class of cyclic di-GMP effectors. Functional genomics of Bacteriology, 192, 1020–1029.
Willsky, G.R. & Malamy, M.H. (1980) Characterization of two genetically separable inorganic phosphate transport systems in *Escherichia coli*. *Journal of Bacteriology*, 144, 356–365.

Yang, C., Huang, T.-W., Wen, S.-Y., Chang, C.-Y., Tsai, S.-F., Wu, W.-F. et al. (2012) Genome-wide PhoB binding and gene expression profiles reveal the hierarchical gene regulatory network of phosphate starvation in *Escherichia coli*. *PLoS One*, 7, e47314.

Zhang, F., Du, Z., Huang, L., Vera Cruz, C., Zhou, Y. & Li, Z. (2013) Comparative transcriptome profiling reveals different expression patterns in *Xanthomonas oryzae pv. oryzae* strains with putative virulence-relevant genes. *PLoS One*, 8, e64267.

Zhang, H.Y., Wei, J.W., Qian, W. & Deng, C.Y. (2020) Analysis of HrpG regulons and HrpG-interacting proteins by ChIP-seq and affinity proteomics in *Xanthomonas campestris*. *Molecular Plant Pathology*, 21, 388–400.

**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section.

**How to cite this article:** Luneau, J.S., Cerutti, A., Roux, B., Carrère, S., Jardinaud, M.-F., Gaillac, A., et al. (2022) *Xanthomonas* transcriptome inside cauliflower hydathodes reveals bacterial virulence strategies and physiological adaptations at early infection stages. *Molecular Plant Pathology*, 23, 159-174. [https://doi.org/10.1111/mpp.13117](https://doi.org/10.1111/mpp.13117)