RESEARCH ARTICLE

Differential accumulation of *Xanthomonas campestris* pv. *campestris* proteins during the interaction with the host plant: Contributions of an in vivo system

Cristiane Santos[1,2], Mariana R. Maximiano[1,3], Daiane G. Ribeiro[1,4], Osmundo B. Oliveira-Neto[1], André M. Murad[1], Octávio L. Franco[2,3,5] and Angela Mehta[1]

[1] Embrapa Recursos Genéticos e Biotecnologia, Brasília, DF, Brazil
[2] Centro de Analises Proteômicas e Bioquímicas, Pós-Graduação em Ciências Genômicas e Biotecnologia, Universidade Católica de Brasília, Brasília, DF, Brazil
[3] Universidade Federal de Juiz de Fora, MG, Brazil
[4] Universidade Paulista, Brasília, DF, Brazil
[5] S-Inova, Pós-Graduação em Biotecnologia, Universidade Católica Dom Bosco, Campo Grande, MS, Brazil

Xanthomonas campestris pv. *campestris* (Xcc) is the causal agent of black rot, a highly destructive disease that affects all brassicas. This work aimed to study the interaction Xcc–*Brassica oleracea* using an in vivo system in an attempt to identify proteins involved in pathogenicity. We used label-free shotgun 2D-nanoUPLC/MS^E to analyze Xcc proteins in three conditions: in the interaction with susceptible (REK) and resistant (REU) plants and in culture medium (control condition). A model of Xcc–susceptible host interaction is proposed and shows that Xcc increases the abundance of several crucial proteins for infection and cell protection. In this study, we also confirmed the differential expression by qPCR analysis of selected genes. This is the first report showing a large-scale identification of proteins in an in vivo host plant condition. Considering that most studies involving phytopathogens are in vitro (growth in culture medium or in plant extract), this work contributes with relevant information related to the plant–pathogen interaction in planta.

Received: March 1, 2017
Revised: April 18, 2017
Accepted: April 28, 2017

Keywords:
Black rot disease / Label-free proteomics / Plant–pathogen interaction

Additional supporting information may be found in the online version of this article at the publisher's web-site

1 Introduction

Xanthomonas campestris is the most complex *Xanthomonas* species due to the numerous host plants it can infect. It is formed by multiple pathovars (more than 140) and causes severe yield losses in agriculture [1, 2]. *X. campestris* pv. *campestris* (Xcc) affects all cruciferous plants and therefore has an important economic impact. This pathogen is disseminated by infected seeds and occurs in all cruciferous producing areas around the world [2, 3].

Genes involved in pathogenicity have been isolated from species of *Xanthomonas*, however the pathogenicity mechanisms are still not elucidated [4]. Regarding Xcc, pathogenicity and virulence genes, which are crucial for disease development, have also been identified such as the *xccR* gene, which might be able to activate the

Correspondence: Dr. Angela Mehta, Embrapa Recursos Genéticos e Biotecnologia, PBI, Av. W/5 Norte Final, CEP 70770–917, Brasília, DF, Brazil
E-mail: angela.mehta@embrapa.br
Fax: +55-61-3340-3658

Abbreviations: **CFU**, colony forming units; **NYG**, nutrient yeast glycerol; **PAMP**, pathogen-associated molecular patterns; **PLGS**, ProteinLynx Global Server; **Xcc**, *Xanthomonas campestris* pv. *campestris*

Colour Online: See the article online to view Figs. 1–3 in colour.

Significance of the study

Black rot disease, caused by *Xanthomonas campestris* pv. *campestris* (Xcc), is a serious problem that affects production of crucifer plants worldwide. Understanding Xcc–host plant interaction is crucial to obtain more efficient strategies to control this disease. Most studies of phytopathogenic bacteria are performed in culture media. In this work, we analyzed the interaction Xcc–*Brassica oleracea* using an in vivo system to identify proteins involved in pathogenicity by label-free shotgun proteomics. We present a model of Xcc–susceptible host interaction and show several proteins differentially abundant in the plant–pathogen interaction in planta.

virulence gene proline iminopeptidase (pip), as well as the genes, Phosphoribosylaminoimidazole-succinocarboxamide synthase (*purC)* and UTP–glucose-1-phosphate uridylyltransferase (*galU)*, whose inactivation led to loss or reduced virulence [5–7]. Although the knowledge of the complete genome sequences of some *Xanthomonas campestris* strains, predicted to encode more than 4.000 proteins, has contributed for the identification of genes involved in infection and host specificity (reviewed in [2]), the question whether these genes are expressed and translated into proteins remains mostly unanswered.

Proteomic studies of *Xanthomonas* species have been performed in order to complement genomic data and identify proteins responsive to different environmental conditions; however, most reports are directed to the cultivation of the bacterium in culture media [8, 9]. Few studies have analyzed *Xanthomonas* protein accumulation in the presence of plant extracts [10, 11] and even fewer under in vivo conditions [12–14]. Recently, high throughput proteomic techniques have been increasingly used to study plant pathogens and have revealed a better view of the changes that occur in protein accumulation [15, 16]. We have previously analyzed the proteome of Xcc during the interaction with *Brassica oleracea*, using an in vivo system and 2DE, however a low number of proteins could be detected and identified [12, 13]. In this study, we have investigated Xcc–*B. oleracea* interaction using the same in vivo system and the shotgun proteomics method 2D-nanoUPLC/MS^E. A model of the susceptible interaction between Xcc and the host plant is proposed, which shows several proteins involved in pathogenicity. Some of these proteins, considered important in the interaction with the host plant, were further analyzed using qPCR.

2 Materials and methods

2.1 In vivo system—infiltration of Xcc in *B. oleracea* leaves

Xcc isolate 51 (Xcc51), obtained from the culture collection of Embrapa Hortaliças was grown on nutrient yeast glycerol (NYG) medium [17]. Young leaves of the *B. oleracea* susceptible (Kenzan-REK) and resistant (União-REU) [18] cultivars were used in this study and maintained in greenhouse conditions. Initially, to determine the moment for bacterial recovery, population dynamics in planta was performed in order to verify the bacterial growth. A total of five biological replicates were performed. Leaf discs of 0.9 cm in diameter were excised at 0, 24, 48, 72, and 120 hours after inoculation (hai), macerated in 1 mL of water and plated onto Petri dishes in appropriate dilutions to count colony forming units (CFU) (Supporting Information Fig. 1). For LC-MS analysis, Xcc51 was precultured in NYG, centrifuged, suspended in 0.85% NaCl (OD_{600} = 0.6) and used to infiltrate leaves of 45 days old plants with a needle-free syringe. The bacterium grown in culture medium NYG (OD_{600} = 0.8) was used as the control condition. The bacterium recovery from the plant was performed according to Mehta and Rosato [19], with some modifications. Briefly, at 48 hai, 15 leaves from three plants were collected and cut in a Petri dish containing distilled water. After 30 min, the solution containing the bacterial cells was filtered under vacuum using filter paper (Millipore®) and again in 5 μm filters (Minisart®). The recovered bacterial cells were used for protein extraction.

2.2 Protein extraction and LC-MS analysis

For the LC-MS analysis, three growth conditions were compared: (1) Xcc recovered from REK; (2) Xcc recovered from REU; and (3) Xcc grown in culture medium (control). For each condition, three biological and three technical replicates were performed, totalizing nine replicates (Supporting Information Fig. 1). Total proteins were extracted according to de Mot & Vanderleyden [20]. Precipitated proteins were solubilized in 50 μL of 50 mM ammonium bicarbonate and prepared for proteomic analysis according to Murad et al. [21], with some modifications. Total proteins (approximately 10 μg) suspended in ammonium bicarbonate were hydrolyzed using trypsin (200 ng) at 37°C. Tryptic peptides were analyzed using a nanoACQUITY™ system (Waters®) with 2D dilution technology coupled to a Synapt G2 HDMS™ mass spectrometer (Waters®) following procedures and settings described in Petriz et al. [22]. The protein identification criteria included the detection of at least one fragment ion per peptide, three peptides per protein, the determination of at least one peptide per protein and the identification of the

protein was allowed with a maximum 4% false-positive discovery rate. Mass spectra were processed and compared to specific *Xanthomonas* spp. in the Uniprot database using ProteinLynx Global Server (PLGS) version 3.0 (Waters®). For the analysis of protein identification and quantification level, the observed intensity measurements were autonormalized and only proteins found in at least two biological replicates were considered. After protein identification, enzymatic classification and gene ontology information were also searched.

2.3 Analysis of gene expression using qPCR

Total RNA was extracted from the bacterial cells recovered from *B. oleracea* susceptible genotype (Kenzan) 48 hai and Xcc grown in culture medium NYG (control group) using the hot acid phenol method [23]. The extracted RNA was treated with Turbo™ DNAse (Applied Biosystems/Ambion) according to the manufacturer's instructions. These samples were submitted to qPCR reaction using the *16S rRNA* (16S Ribossomal RNA) primer to verify the absence of genomic DNA. Approximately 1 μg of total RNA was used to synthesize cDNA using the *Go Script*™ Reverse Transcription System (Promega) according to manufacturer's instructions. Specific primers (Supporting Information Table 1) were designed using Primer 3 program [24].

A total of nine genes encoding the proteins highlighted in our model, considered important for the pathogenicity of Xcc (susceptible interaction), were selected for qPCR: Kad (Q3BPM9, *adk*); CarB (P58942, *carB*); AcP (P63446, *acpP*); FabH (Q8PBV1, *fabH*); ArgD (Q8PH31, *argD*); FabV (Q8PE66, *fabV*); Clp (Q4UZF6, *clp*); ClpX (Q8PNI4, *clpX*); and Ssb (Q8PIJ2, *ssb*).

The qPCR experiments were performed in the thermal cycler 7300 Real-Time PCR System (Applied Biosystems). The reaction consisted of Fast SYBR® Green Master Mix (Applied Biosystems), 0.2 μM of each primer (forward and reverse) and 2 μL single-stranded cDNA (diluted 20X) corresponding to each sample. The PCR program used was a step at 95°C for 10 min to activate the *Taq* polymerase enzyme (hot start), 95°C for 15 s, 60°C for 60 s repeated for 40 cycles. The denaturation curve - "melt curve" (95°C for 15 s, 60°C for 60 s, and increased 0.3°C up to 95°C) was held after the end of the amplification to verify the presence of nonspecific products or primer dimmers. The raw data of fluorescence for all runs were imported into the Real-time PCR Miner software [25] in order to determine the Cq value and the PCR efficiency. The relative expression (fold change) and statistics were performed using REST software [26]. All experiments were performed with three biological and three technical replicates. For normalization, the *16S rRNA* (16S Ribosomal RNA) [27] and *iroN* (TonB-dependent receptor) genes were used. These genes were selected based on analyses of the geNorm software [28].

3 Results and discussion

3.1 Population dynamics of Xcc and symptom development

In order to determine the sampling point with the highest number of Xcc cells recovered from the plant, a population dynamics study was performed (Fig. 1). The statistic analysis confirmed that in both cultivars the time point with the highest number of cells was at 48 hai (REK 7.3 $\log_{10}$CFU/cm^2 and REU 7.1 $\log_{10}$ CFU/cm^2). A decrease in the population could be observed at 72 hai (Fig. 1) and therefore, 48 hai was selected as the sampling point for the proteomic analysis for both REK and REU interactions. The symptoms were also compared and began at 48 hai on both cultivars. In REK, injuries were more pronounced, with a higher affected area beyond the infiltration point showing chlorotic lesions, necrotic regions, and gelatinous consistency of infected area (Fig. 1A–C). On the other hand, lesions were dry and contained in REU cultivar (Fig. 1D–F). These results show a clear difference in disease development in both cultivars and confirm previous data obtained by our group [13].

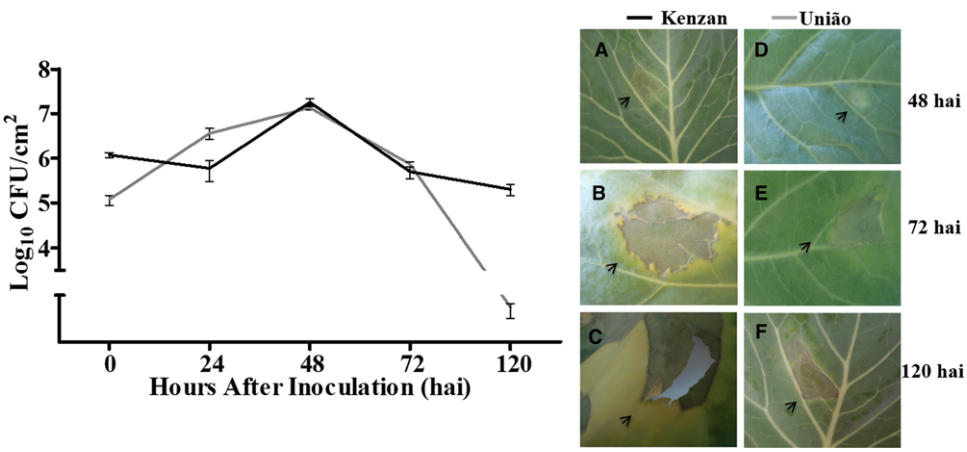

Figure 1. Comparison of bacterial growth in infiltrated leaves of Kenzan and União with Xcc at five different time points; vertical bars indicate standard error of three biological replicates. Tukey's multiple comparisons test and two-way RM ANOVA analyzed p value ≤ 0.05. On the right, *B. oleracea* leaves of Kenzan and União inoculated with *X. campestris* pv. *campestris* showing the onset of necrosis at 48 hai. The black arrows indicate the infiltrated region with necrosis.

3.2 Proteomic analysis

In this study, a comparative analysis of proteins accumulated in vivo during the interaction of Xcc and the susceptible (REK) and resistant (REU) brassica plants was performed to identify proteins, which may be important for pathogenicity. The data obtained by 2D-nanoUPLC/MSE was processed and submitted to analysis in the *PLGS* platform (Supporting Information Table 2, spreadsheets 1–5). More than 4000 peptides were obtained and were grouped into approximately 380 proteins. A lower number of proteins was identified in the in vivo conditions when compared to growth in the rich medium NYG. Probably, due to the stressful environment during contact with the host plant, the bacterium needs to save energy and therefore reduce growth. Similar results have been reported earlier using 2DE [29]. Moreover, it is probably necessary to synthesize several proteins for energy production and uptake.

Plant proteins were also identified in the in vivo samples (Supporting Information Table 3). It was interesting to observe a lower diversity of the bacterial proteins identified in REU as well as a higher amount of plant proteins in this sample. Similar results were obtained by Villeth et al. [13] when analyzing Xcc recovered from the resistant plant (REU) by 2DE. It seems that the presence of more plant proteins in REU is indeed a response of the resistant plant and not a technical artifact, especially since these results were consistent in all biological replicates by using two proteomic techniques. The presence of plant proteins may be a limitation of the in vivo approach, especially since they could compete during detection and could account for the lower number of bacterial proteins detected due to signal suppression when compared to culture medium growth. However, this method is the closest to the naturally occurring infection of the plant and has proven useful for a large-scale screening of protein abundance.

For the proteomic analysis, comparisons between REU and the control condition (REU:control), REK and the control condition (REK:control) and between REK and REU (REK:REU) were performed. Repeated proteins (same protein product with different accession numbers showing identical sequences) were considered only once. In the comparison REU:control, 108 differential proteins were identified out of 121 proteins, including 80 exclusive to the control. A total of 14 proteins were increased and 14 decreased in REU (Supporting Information Table 2 spreadsheet 3). In the comparison REK:control, 105 differential proteins were identified out of 127, including 46 exclusive to the control, 34 increased and 22 decreased in REK. Three proteins were exclusive to REK (Supporting Information Table 2 spreadsheet 4): Acetylornithine aminotransferase (ArgD/Q8PH31) and Ketol-acid reductoisomerase (IlvC/Q8PH09 and IlvC/Q3BPK3). Interestingly, IlvC was also detected in the control condition under different accession numbers (Q4UYF7; B0RP32; Q8P5L5). The alignment of all five sequences revealed several differences between IlvC identified in REK and IlvC identified in the control, indicating that they represent different protein species. Similarly, other proteins also showed this behavior and are indicated by an asterisk in Supporting Information Table 2. These results suggest that Xcc may have copies of the same gene, which have different expression patterns. The third comparison, performed between REK and REU (REK:REU), revealed 80 proteins in total, 17 of which showed differential abundance, including four increased and 13 decreased in REK. A total of 35 proteins were unique to REK (Supporting Information Table 2 spreadsheet 5).

In order to characterize the function of the identified proteins, a classification according to GO was performed (biological process and molecular function), using the data from UniProt (Source: UniProtKB-HAMAP or InterPro). The GO analysis revealed that most proteins with increased abundance in REK (61%), when compared to the control, were related to cellular metabolism (citrate cycle, glycolysis/gluconeogenesis, oxidative phosphorylation, other carbohydrate metabolism, and pentose phosphate pathway) (Supporting Information Fig. 2A). Moreover, a large number of proteins were associated with protein synthesis corresponding to 15% (transcription and translation factors as well as ribosomal proteins). DNA replication and maintenance of cellular activity have been reported as essential for colonization and dissemination of Xcc in infected tissue [9]. Some studies have reported the possibility of an increase in metabolism proteins during the infection process, which could be involved in bacterial pathogenicity [30]. Nevertheless, the functions of these genes in bacterial physiology and their relations in metabolism, catabolism, and pathogenicity are not well understood.

Most proteins with reduced abundance in this interaction were components of translational machinery (59% ribosomal proteins, followed by 18% chaperones and 14% elongation and translation factors) (Supporting Information Fig. 2B). Bacterial growth is mainly dependent on the ribosome and related proteins; therefore protein synthesis is crucial for bacterial biomass formation [31]. These results may be explained by the reduction in bacterial growth, probably due to restricted nutrient availability in the plant tissue, when compared to culture medium conditions.

When REU:control were compared, it was interesting to note that the functional groups of the proteins identified were very similar to that of REK:control (Supporting Information Fig. 2C–D). As expected, several unique proteins were identified in the control condition when compared to REK (46) and REU (80). Most of these proteins are involved in energy acquisition, primary and secondary metabolism, cellular growth, and macromolecule synthesis. In culture medium conditions, temperature and nutrients are optimal for growth and development of the bacteria and therefore the abundance in metabolism proteins is expected and has been identified in other studies [9, 32]. Some uncharacterized proteins were identified in the control condition and their function is yet unknown.

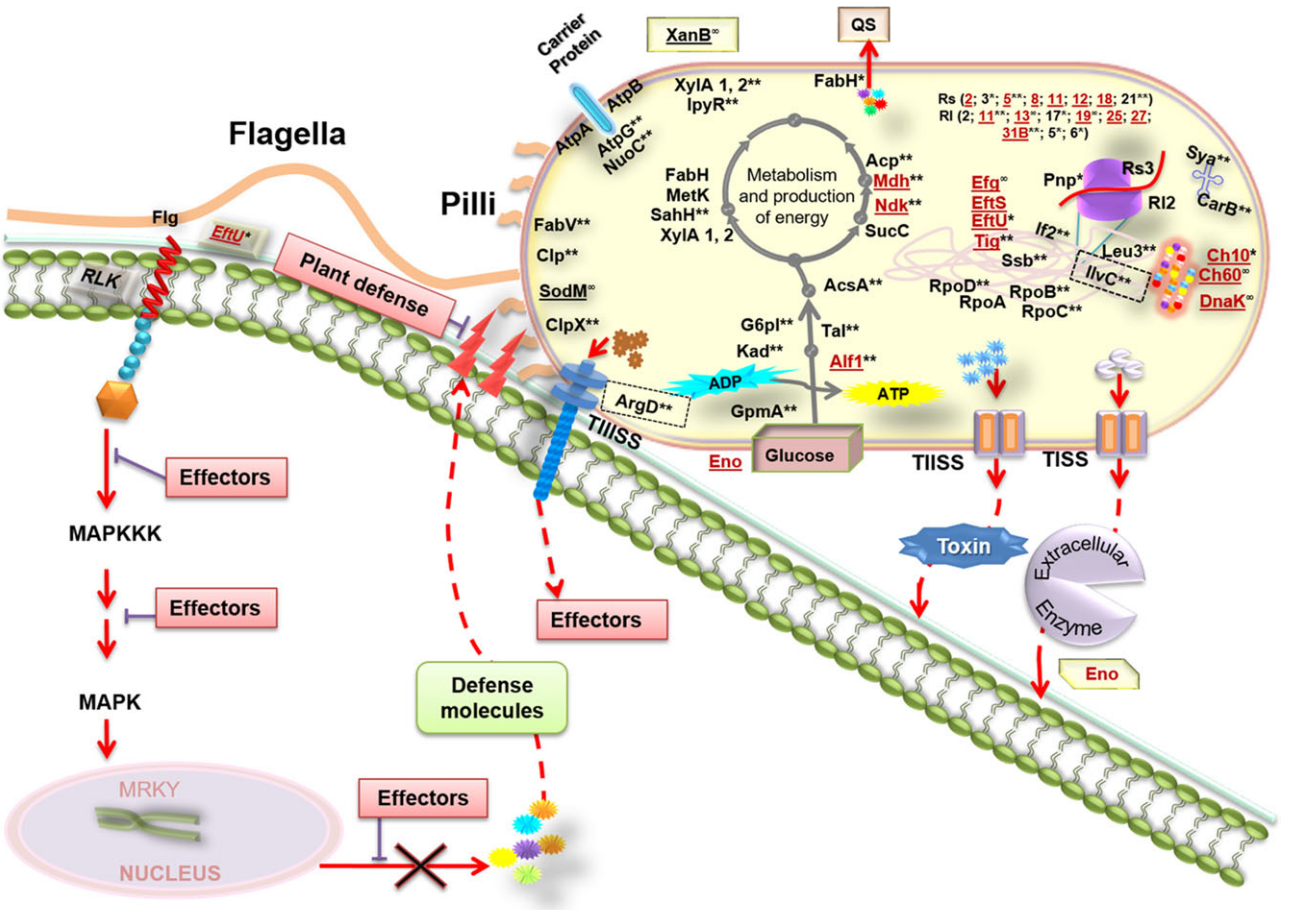

Figure 2. Model of susceptible Xcc–host interaction. Proteins in black and red (underlined) indicate increased and decreased abundance, respectively, in REK when compared to the control. Proteins in dotted rectangles indicate unique proteins in REK compared to the control. (**) Exclusive protein in REK when compared to REU; (*/∞) proteins with increased and decreased abundance, respectively, in REK when compared to REU; (QS) quorum sensing; (RKL) receptor-like kinase; (TISS) type I secretion system; (TIISS) type II secretion system; (TIIISS) type III secretion system.

In the comparison REK:REU, most differential proteins with increased abundance were involved in protein synthesis and assembly (Supporting Information Fig. 2E–F). These results are in accordance with the successful colonization of the plant tissue during a susceptible interaction, which requires a more efficient functioning of the protein synthesis apparatus. Moreover, the bacterial cells are probably in a less hostile environment in the susceptible plant when compared to the resistant. Chaperones were also identified and some were increased while others were decreased in REK. Chaperones are primarily involved in protein assembly and folding, however they may perform several other functions in the cell, such as transport of precursor proteins to and across biological membranes, modulation of the oligomeric state of protein complexes and facilitation of protein degradation [33]. The multiple functions performed by different chaperones may explain the diversity in their abundance during Xcc–plant interaction. Several proteins unique to REK were also observed and seem to be important for pathogenicity and host colonization. These proteins are discussed in the model below.

3.2.1 Model for Xcc–host plant interaction

Here we discuss proteins, which were considered the most relevant in the interaction with the plant, based on their function. Some of these proteins were selected for qPCR analysis. We propose here a model for Xcc–host plant interaction in vivo, considering the proteins detected in the REK:control comparison (Fig. 2 and Supporting Information Table 4). Some proteins were also identified in the other comparisons and therefore in some cases, a general discussion regarding their role in all three conditions is presented.

The model illustrates the first line of defense of the host plant, a basal response mediated by the recognition of pathogen-associated molecular patterns (PAMPs), such as flagelin proteins. The recognition of PAMPs by the plant results in an immunity response known as PTI (PAMP-triggered immunity). In this study, in all compared conditions (REU:control, REK:control, and REK:REU), the expression of some elongation factors (G, Ts, and Tu) was reduced, however, in REK:REU, EF-Tu-1, and EF-Tu-2 (*tfu1, tfu2*) were

increased in REK. Elongation factors are among the most abundant proteins in bacterial cells and are highly conserved [34]. Studies have shown that EF-Tu proteins have complex and multiple functions and can act as PAMPs, suggesting that the recognition of EF-Tu by the plant occurs by an EF-Tu receptor, which results in the activation of defense responses in the plant [35, 36]. The differential abundance of EF-Tu-1 and EF-Tu-2 in REK may be related to the ability of Xcc to overcome plant defense and establish infection.

When the plant recognizes PAMPs by surface-localized plasma membrane pattern recognition repeat (PRR), a signaling pathway is triggered (PTI), including the production of ROS, considered the first line of defense, formed in an attempt to contain the pathogen. Xcc then reacts against the ROS produced by the plant and tries to create a favorable environment for survival and growth by increasing the production of superoxide dismutase (sodA). Interestingly, sodA was increased in REK:control and REU:control and reduced in REK:REU. These results indicate that in the first two comparisons, sodA is being produced to overcome plant defense response and in the third (REK:REU), sodA is reduced in REK since in REU, Xcc needs to produce higher amounts of this protein in order to try to establish infection, probably due to a higher production of ROS in REU. Similar results were obtained for XanB, involved in polysaccharide production, probably for cellular protection. XanB showed the same expression profile as sodA, which can also be explained as an attempt of Xcc to preserve survival in a more hostile environment due to a more pronounced defense response in REU.

A high number of proteins involved in metabolism and production of energy were identified and are shown in the model, including, Succinyl-CoA ligase [ADP-forming] subunit beta (SucC), Malate dehydrogenase (Mdh), Acyl carrier proteins (Acp), S-adenosylmethionine synthase (MetK), Xylose isomerase 1 and 2 (XylA 1; XylA2), Enoyl-[acyl-carrier-protein] reductase (FabV), 3-oxoacyl-[acyl-carrier-protein] synthase 3 (FabH), Single-stranded DNA-binding protein (Ssb) and Adenylate kinase (Kad). These results suggest that Xcc increases the abundance these proteins to colonize the host tissue. Two of these proteins were selected for qPCR: Acyl carrier protein and Enoyl-(acyl-carrier-protein) reductase (FabV and FabH). Some of these proteins may have additional functions and may be involved in pathogenicity. FabH (unique to REK), for example, is an enzyme important for fatty acid biosynthesis and has been associated with regulation of virulence factors [37]. The *acpP* and *fabV* genes are also important for fatty acid biosynthesis, phospholipid biosynthesis, lipopolysaccharide biosynthesis, activation of prohemolysin, and membrane-derived oligosaccharide biosynthesis [38–41].

During the interaction with the host plant, the bacterium also produces important proteins for growth, such as DNA directed RNA polymerase subunits alpha and beta (RpoA and RpoB, respectively). The model shows other identified proteins related to glycolysis (enolase and 2, 3-biphosfoglycerate), as well as cellular division and protein synthesis (DNA-directed RNA polymerase subunits, alpha, beta, and beta' (RpoC); RNA polymerase sigma factor (RpoD), tRNA-directed proteins (CarB, analyzed by qPCR), and mRNA-directed proteins (30S ribosomal proteins S3 (Rs3); polyribonucleotide nucleotidyltransferase (Pnp); 50L ribosomal protein L2, (Rl2)), which are also increased. However, a lower growth rate is maintained in planta, when compared to the culture medium condition, which is in accordance with the decreased abundance of several proteins associated with ribosome assembly, such as 30S and 50L ribosomal proteins (Fig. 2, red underlined). The Trigger factor (Tig) and chaperone proteins 60 kDa (Ch60), 10 kDa (Ch10), and DnaK, important for protein folding, are also reduced during infection. The reduction in the abundance of chaperones in vivo may be related to the compromised translational apparatus (ribosomal proteins). Interestingly, ClpX (analyzed by qPCR), also involved in protein folding, was exclusive to REK in the REK:REU comparison and exclusive to the control in REU:control comparison (Supporting Information Table 2). These results show that this protein is produced in REK and in the control condition in a similar way, however in REU it is not produced. It seems this protein is probably affected by plant defense in the resistant plant.

In order to identify variations in the environment, bacteria use extracellular signals such as nutrient availability (carbon, ions, phosphorous, sulfur, etc.) and environmental conditions (pH, temperature, oxygen availability, oxidative stress, etc.). Bacteria have complex mechanisms to respond to these changes by modulating proteins related to transcription regulation. In our study, we observed that Xcc increases such proteins including RpoA, RpoB, RpoC, and RpoD, also involved in protein synthesis as mentioned above. The increase in abundance of these proteins in Xcc may play an important role in adaptation and colonization of the plant cell, using the host tissue as energy source to survive, multiply, and produce effectors in order to overcome plant defenses and cause the disease. Clp (CRP [cyclic AMP receptor protein]-like protein), analyzed by qPCR, is also highly important for pathogenicity and is produced during plant infection. Clp is a transcription factor that regulates the expression of a high number of genes involved in pathogenicity in xanthomonads [42]. It has also been reported that the deletion of *clp* in Xcc significantly decreases expression of virulence-associated functions [42]. As reported by He et al. [42], Clp can function as (1) global regulator in DSF (Diffusible Signal Factor) signaling pathway to regulate DSF-controlled genes/functions responsible for virulence, such as genes encoding extracellular enzymes, exopolysaccharides (EPS) biosynthesis, and flagellum synthesis and (2) DSF-independent modulation of biofilm formation. It was interesting to detect Clp as exclusive to the control in the REU:control comparison, which may be related to biofilm formation or to exopolysaccharides biosynthesis. Similarly, engXCA, which is an edoglucanase whose expression may also be controlled by DSF [43], was also exclusive to the control in the same comparison. As

reported by Wang et al. [44], DSF molecules are also produced in rich media (YEB and NYG), which may explain these findings.

Another mechanism to circumvent the plant defense is the increase in abundance of membrane proteins for ion transport such as ATP synthase subunit alpha (AtpA), beta (AtpB), and gamma chain (AtpG), as well as proteins involved in cellular repair including DNA-directed RNA polymerase subunit alpha, beta and beta', 3-isopropylmalate dehydrogenase (Leu3), Ketol-acid reductoisomerase (IlvC). The bacterial cell has the ability to detect metabolites, essential for amino acid synthesis that are in the extracellular space and direct them into the cell, which is a way of saving energy since the bacterium does not have to produce them. Indeed, we have identified transport proteins involved in oxidative phosphorylation, ion, and proton transport (AtpB, AtpD, and AtpG) as well as transport proteins directly associated with respiratory chain in the synthesis of ATP.

Although the proteins mentioned above may be related to the capacity of the bacterium to infect the host plant, the main pathogenicity mechanism of *Xanthomonas* involves the production of effectors, which are delivered into the plant cell through the Type III Secretion System (TIIISS). This mechanism, developed during evolution to overcome initial plant defense (PTI), results in effector-trigged susceptibility [45]. In this study, we have identified proteins, such as acetylornithine (ArgD), which are delivered by this system and were unique to REK, when compared to REU. ArgD is part of a group of proteins involved in the biosynthesis of ornithine, a constituent of faseolotoxin, which is a type of phototoxin [46]. Faseolotoxin can cause arginine deficiency, chlorosis, and growth inhibition in the host plant [47]. In silico studies have shown that the same region that encodes this protein also encodes a family of transcription factors (AraC), that regulate the expression of a group of harpins and other effectors [46]. Harpins are proteins secreted by Gram-negative bacteria through the TIIISS, and are capable of triggering a hypersensitive response in plants [48]. These proteins can be directed to the intercellular spaces in the plant tissue and trigger an innate immune response in the plant [49].

As mentioned earlier, a total of nine genes were selected for qPCR in order to complement the proteomic data. Since our model focused on the susceptible interaction, we analyzed gene expression using only REK samples. The amplification efficiencies were determined individually for each sample by the Miner software and were above 90% for all genes evaluated (Supporting Information Table 1). The expression analysis confirmed the upregulation of all selected genes (Fig. 3). Therefore, we can conclude that these genes, important directly or indirectly for Xcc colonization of the host plant, are being regulated in a similar pattern as proteins during the interaction with the host plant, and could be used as future targets for knockout studies to confirm their role in pathogenicity.

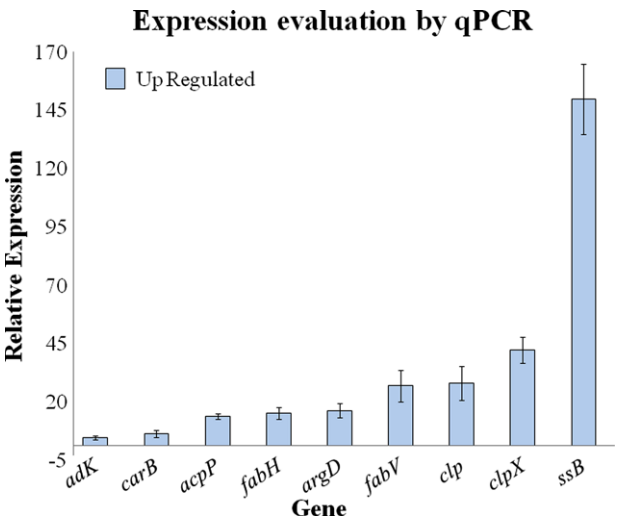

Figure 3. Relative expression quantification of genes involved in the pathogenicity process of Xcc in planta. Each value represents the mean of ± SE of three independent experiments ($p < 0.05$).

4 Concluding remarks

Although Xcc is a well-studied bacterium, most of the analyses performed report the characterization of genes expressed in culture media conditions [8, 9]. In our work, we have analyzed protein abundance of Xcc–*B. oleracea* interaction using an in vivo system and have identified several proteins involved in the interaction with the plant. The results obtained showed that Xcc increases the abundance of pathogenicity proteins, all important for plant infection and plant colonization. On the other hand, Xcc saves energy reducing the abundance of ribosomal proteins and consequently protein synthesis and growth. Since plant proteins were also identified in the in vivo samples, a comparison of the identified proteins to *Brassica* database was also performed (Supporting Information Table 3). Among the plant proteins detected in REK and REU were proteins involved in ethylene sensing and induction of jasmonate. It seems that jasmonic acid and ethylene pathways are activated during the interaction with the pathogen. Several defense and stress-related proteins were observed, especially in REU, such as lipoxygenase, annexins, and apocitocrome f, as well as antimicrobial compounds (phitoalexins). Interestingly, proteins associated to photosystem were also identified in REU (I P700; II CP43; II; Q(B); CP47). As observed earlier by Villeth et al. [13], these results indicate that the plant tries to minimize the damage caused by Xcc by increasing important proteins for the regulation of photosynthesis. Although we have highlighted some plant proteins in this study, a deeper view can only be obtained by the analysis of the plant proteome. Overall, the results obtained in this study show a comprehensive view of the Xcc–host plant interaction. A clear increase in pathogenicity proteins in the susceptible

and resistant interactions was observed. Future studies will be performed in order to verify the direct role of these proteins in pathogenicity and contribute for the development of more efficient strategies for black rot disease control. This is the first report showing a large scale identification of proteins in an in vivo condition.

This work was sponsored by Embrapa, CAPES, Ministério da Ciência e Tecnologia – MCT, CNPq, FAPDF and FUNDECT. We acknowledge the Laboratory of Mass Spectrometry of Embrapa Recursos Genéticos e Biotecnologia for the support in the mass spectrometry analysis.

The authors have no conflict of interest to declare.

5 References

[1] Vauterin, L., Hoste, B., Kersters, K., Swings, J., Reclassification of xanthomonas. *Int. J. Syst. Evol. Microbiol.* 1995, *45*, 472–489.

[2] Ryan, R. P., Vorhölter, F.-J., Potnis, N., Jones, J. B. et al., Pathogenomics of Xanthomonas: understanding bacterium–plant interactions. *Nat. Rev. Microbiol.* 2011, *9*, 344–355.

[3] Vorhölter, F. J., Thias, T., Meyer, F., Bekel, T. et al., Comparison of two *Xanthomonas campestris* pathovar campestris genomes revealed differences in their gene composition. *J. Biotechnol.* 2003, *106*, 193–202.

[4] da Silva, A. C. R., Comparison of the genomes of two Xanthomonas pathogens with differing host specificities. *Nature* 2002, *417*, 459–463.

[5] Yuan, Z., Wang, L., Sun, S., Wu, Y., Qian, W., Genetic and proteomic analyses of a *Xanthomonas campestris* pv. campestris purC mutant deficient in purine biosynthesis and virulence. *J. Genet. Genomics* 2013, *40*, 473–487.

[6] Liao, C.-T., Du, S.-C., Lo, H.-H., Hsiao, Y.-M., The galU gene of *Xanthomonas campestris* pv. campestris is involved in bacterial attachment, cell motility, polysaccharide synthesis, virulence, and tolerance to various stresses. *Arch. Microbiol.* 2014, *196*, 729–738.

[7] Zhang, L., Jia, Y., Wang, L., Fang, R., A proline iminopeptidase gene upregulated in planta by a LuxR homologue is essential for pathogenicity of *Xanthomonas campestris* pv. campestris. *Mol. Microbiol.* 2007, *65*, 121–136.

[8] Watt, S. A., Wilke, A., Patschkowski, T., Niehaus, K., Comprehensive analysis of the extracellular proteins from *Xanthomonas campestris* pv. *campestris* B100. *Proteomics* 2005, *5*, 153–167.

[9] Soares, M. R., Facincani, A. P., Ferreira, R. M., Moreira, L. M. et al., Proteome of the phytopathogen *Xanthomonas citri* subsp. citri: a global expression profile. *Proteome Sci.* 2010, *8*, 55.

[10] Tahara, S. T., Mehta, A., Rosato, Y. B., Proteins induced by *Xanthomonas axonopodis* pv. passiflorae with leaf extract of the host plant (*Passiflorae edulis*). *Proteomics* 2003, *3*, 95–102.

[11] Mehta, A., Rosato, Y. B., Differentially expressed proteins in the interaction of *Xanthomonasaxonopodis* pv. *citri* with leaf extract of the host plant. *Proteomics* 2001, *1*, 1111–1118.

[12] Andrade, A. E., Silva, L. P., Pereira, J. L., Noronha, E. F. et al., In vivo proteome analysis of *Xanthomonas campestris* pv. *campestris* in the interaction with the host plant *Brassica oleracea*. *FEMS Microbiol. Lett.* 2008, *281*, 167–174.

[13] Villeth, G. R., Reis Jr, F. B., Tonietto, A., Huergo, L. et al., Comparative proteome analysis of *Xanthomonas campestris* pv. *campestris* in the interaction with the susceptible and the resistant cultivars of *Brassica oleracea*. *FEMS Microbiol. Lett.* 2009, *298*, 260–266.

[14] Wang, Y., Kim, S. G., Wu, J., Huh, H. H. et al., Secretome analysis of the rice bacterium *Xanthomonas oryzae* (Xoo) using in vitro and in planta systems. *Proteomics* 2013, *13*, 1901–1912.

[15] Sidhu, V. K., Vorhölter, F.-J., Niehaus, K., Watt, S. A., Analysis of outer membrane vesicle associated proteins isolated from the plant pathogenic bacterium *Xanthomonas campestris* pv. *campestris*. *BMC Microbiol.* 2008, *8*, 87.

[16] Gonzalez-Fernandez, R., Aloria, K., Arizmendi, J. M., Jorrin-Novo, J. V., Application of label-free shotgun nUPLC–MSe and 2-DE approaches in the study of *Botrytis cinerea* Mycelium. *J. Proteome Res.* 2013, *12*, 3042–3056.

[17] Daniels, M., Barber, C., Turner, P., Sawczyc, M. et al., Cloning of genes involved in pathogenicity of *Xanthomonas campestris* pv. *campestris* using the broad host range cosmid pLAFR1. *EMBO J.* 1984, *3*, 3323–3328.

[18] Giordano, L. D. B., Silva, N., Buso, J. A., União: nova cultivar de repolho para o verão. *Hortic. Bras.* 1988, *6*, 39.

[19] Mehta, A., Rosato, Y. B., A simple method for in vivo expression studies of *Xanthomonas axonopodis* pv. *citri*. *Curr. Microbiol.* 2003, *47*, 400–403.

[20] De Mot, R., Vanderleyden, J., Application of two-dimensional protein analysis for strain fingerprinting and mutant analysis of *Azospirillum* species. *Can. J. Microbiol.* 1989, *35*, 960–967.

[21] Murad, A. M., Souza, G. H. M. F., Garcia, J. S., Rech, E. L., Detection and expression analysis of recombinant proteins in plant-derived complex mixtures using nanoUPLC-MSE. *J. Sep. Sci.* 2011, *34*, 2618–2630.

[22] Petriz, B. A., Almeida, J. A., Gomes, C. P. C., Pereira, R. W. et al., NanoUPLC/MSE proteomic analysis reveals modulation on left ventricle proteome from hypertensive rats after exercise training. *J. Proteomics* 2015, *113*, 351–365.

[23] Schulte, R., Bonas, U., Expression of the *Xanthomonas campestris* pv. *vesicatoria* hrp gene cluster, which determines pathogenicity and hypersensitivity on pepper and tomato, is plant inducible. *J. Bacteriol.* 1992, *174*, 815–823.

[24] Untergasser, A., Cutcutache, I., Koressaar, T., Ye, J. et al., Primer3—new capabilities and interfaces. *Nucleic Acids Res.* 2012, *40*, e115–e115.

[25] Srivastava, G. P., Hanumappa, M., Kushwaha, G., Nguyen, H. T., Xu, D., Homolog-specific PCR primer design for profiling splice variants. *Nucleic Acids Res.* 2011, *39*, e69.

[26] Pfaffl, M. W., Horgan, G. W., Dempfle, L., Relative expression software tool (REST) for group-wise comparison and

statistical analysis of relative expression results in real-time PCR. *Nucleic Acids Res.* 2002, *30*, e36.

[27] Martins, P. M. M., Machado, M. A., Silva, N. V., Takita, M. A., de Souza, A. A., Type II toxin-antitoxin distribution and adaptive aspects on Xanthomonas genomes: focus on *Xanthomonas citri*. *Front. Microbiol.* 2016, *7*, 652.

[28] Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B. et al., Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol.* 2002, *3*, research0034.0031-research0034.0011.

[29] Andrade, A. E., Silva, L. P., Pereira, J. L., Noronha, E. F. et al., In vivo proteome analysis of *Xanthomonas campestris* pv. *campestris* in the interaction with the host plant *Brassica oleracea*. *FEMS Microbiol. Lett.* 2008, *281*, 167–174.

[30] Qian, W., Jia, Y., Ren, S.-X., He, Y.-Q. et al., Comparative and functional genomic analyses of the pathogenicity of phytopathogen *Xanthomonas campestris* pv. campestris. *Genome Res.* 2005, *15*, 757–767.

[31] Klumpp, S., Scott, M., Pedersen, S., Hwa, T., Molecular crowding limits translation and cell growth. *Proc. Natl. Acad. Sci.* 2013, *110*, 16754–16759.

[32] Lima, W. C., Paquola, A. C. M., Varani, A. M., Van Sluys, M.-A., Menck, C. F. M., Laterally transferred genomic islands in Xanthomonadales related to pathogenicity and primary metabolism. *FEMS Microbiol. Lett.* 2008, *281*, 87–97.

[33] Pfanner, N., Protein folding: who chaperones nascent chains in bacteria? *Curr. Biol.* 1999, *9*, R720–R724.

[34] Filer, D., Furano, A. V., Portions of the gene encoding elongation factor Tu are highly conserved in prokaryotes. *J. Biol. Chem.* 1980, *255*, 728–734.

[35] Kunze, G., Zipfel, C., Robatzek, S., Niehaus, K. et al., The N terminus of bacterial elongation factor Tu elicits innate immunity in Arabidopsis plants. *Plant Cell* 2004, *16*, 3496–3507.

[36] Zipfel, C., Kunze, G., Chinchilla, D., Caniard, A. et al., Perception of the bacterial PAMP EF-Tu by the receptor EFR restricts agrobacterium-mediated transformation. *Cell* 2006, *125*, 749–760.

[37] Williams, P., Quorum sensing, communication and cross-kingdom signalling in the bacterial world. *Microbiology* 2007, *153*, 3923–3938.

[38] Tang, L., Weissborn, A. C., Kennedy, E. P., Domains of *Escherichia coli* acyl carrier protein important for membrane-derived-oligosaccharide biosynthesis. *J. Bacteriol.* 1997, *179*, 3697–3705.

[39] Thomas, J., Cronan, J. E., The enigmatic acyl carrier protein phosphodiesterase of *Escherichia coli* GENETIC AND ENZYMOLOGICAL CHARACTERIZATION. *J. Biol. Chem.* 2005, *280*, 34675–34683.

[40] Chan, D. I., Vogel, H. J., Current understanding of fatty acid biosynthesis and the acyl carrier protein. *Biochem. J.* 2010, *430*, 1–19.

[41] Janßen, H. J., Steinbüchel, A., Fatty acid synthesis in *Escherichia coli* and its applications towards the production of fatty acid based biofuels. *Biotechnol. Biofuels* 2014, *7*, 7–7.

[42] He, Y. W., Xanthomonas campestris cell–cell communication involves a putative nucleotide receptor protein Clp and a hierarchical signalling network. *Mol. Microbiol.* 2007, *64*, 281–292.

[43] Barber, C. E., Tang, J. L., Feng, J. X., Pan, M. Q. et al., A novel regulatory system required for pathogenicity of *Xanthomonas campestris* is mediated by a small diffusible signal molecule. *Mol. Microbiol.* 1997, *24*, 555–566.

[44] Wang, L.-H., He, Y., Gao, Y., Wu, J. E. et al., A bacterial cell–cell communication signal with cross-kingdom structural analogues. *Mol. Microbiol.* 2004, *51*, 903–912.

[45] Jones, J. D. G., Dangl, J. L., The plant immune system. *Nature* 2006, *444*, 323–329.

[46] Zhang, Y. X., Patil, S. S., The phtE locus in the phaseolotoxin gene cluster has ORFs with homologies to genes encoding amino acid transferases, the AraC family of transcriptional factors, and fatty acid desaturases. *Mol. Plant-Microbe Interact.* 1997, *10*, 947–960.

[47] Patil, S. S., Toxins produced by phytopathogenic bacteria. *Annu. Rev. Phytopathol.* 1974, *12*, 259–279.

[48] Choi, M.-S., Kim, W., Lee, C., Oh, C.-S., Harpins, multifunctional proteins secreted by gram-negative plant-pathogenic bacteria. *Mol. Plant-Microbe Interact.* 2013, *26*, 1115–1122.

[49] Lee, J., Klessig, D. F., Nürnberger, T., A harpin binding site in tobacco plasma membranes mediates activation of the pathogenesis-related gene HIN1 independent of extracellular calcium but dependent on mitogen-activated protein kinase activity. *Plant Cell* 2001, *13*, 1079–1093.

