RESEARCH LETTER

Comparative proteome analysis of *Xanthomonas campestris* pv. *campestris* in the interaction with the susceptible and the resistant cultivars of *Brassica oleracea*

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Received 6 February 2009; accepted 1 July 2009.
Final version published online 3 August 2009.

DOI:10.1111/j.1574-6968.2009.01728.x

Editor: Juan Imperial

Keywords
Xanthomonas; proteome; 2-DE; MS; *in vivo* expression.

Abstract

Black rot of cruciferous plants, caused by *Xanthomonas campestris* pv. *campestris*, causes severe losses in agriculture around the world. This disease affects several cultures, including cabbage and broccoli, among others. Proteome studies of this bacterium have been reported; however, most of them were performed using the bacterium grown under culture media conditions. Recently, we have analyzed the proteome of *X. campestris* pv. *campestris* during the interaction with the susceptible cultivar of *Brassica oleracea* and several proteins were identified. The objective of the present study was to analyze the expressed proteins of *X. campestris* pv. *campestris* during the interaction with the resistant cultivar of *B. oleracea*. The bacterium was infiltrated in the leaves of the resistant plant and recovered for protein extraction and two-dimensional electrophoresis. The protein profile was compared with that of the bacterium isolated from the susceptible host and the results obtained revealed a group of proteins exclusive to the resistant interaction. Among the proteins identified in this study were plant and bacterium proteins, some of which were exclusively expressed during the resistant interaction.

Introduction

Agriculture productivity is highly affected by several diseases caused by species of the genus *Xanthomonas*, which results in substantial losses in several economically important crops. Black rot, caused by *Xanthomonas campestris* pv. *campestris*, is considered to be one of the most destructive diseases of cruciferous plants. This disease affects all crucifer-producing areas around the world and causes losses in the production and quality of the products. Black rot can be controlled using healthy plant material including seeds and transplants and by adopting cultural practices that limit bacterial spread. The most efficient form of disease control is using resistant cultivars; however, only a small number of useful sources of resistance are available.

Proteomic analysis represents a valuable approach to study differential expression because actual differences in protein abundance at the time of sampling can be observed. Moreover, different forms of the same protein can be distinguished. The proteomic approach is a very useful method for understanding physiological processes and for the identification of the functions of proteins expressed under a given condition (Mehta *et al.*, 2008). Recently, the proteomes of phytopathogens such as *Xylella fastidiosa* (Smolka *et al.*, 2003; Martins *et al.*, 2007) and *X. campestris* (Watt *et al.*, 2005; Chung *et al.*, 2007) have been described. However, most of these studies were performed under culture media conditions and very few addressed the variation in the bacterial proteome upon the establishment of the phytopathogenic interaction. One interesting approach is the use of plant extract to mimic *in planta* conditions. Some studies have been performed in *Xanthomonas axonopodis* using this strategy, and genes and proteins differentially expressed were identified (Mehta & Rosato, 2001; Tahara *et al.*, 2003). However, one of the major drawbacks of this approach is the determination of the ideal plant extract concentration to be used for bacterial growth.

In a recent study, we have reported several proteins of *X. campestris* pv. *campestris* in the *in vivo* interaction with the susceptible cultivar of *Brassica oleracea* (Andrade *et al.*, 2008). In the present work, we have used the same approach and compared the proteome of *X. campestris* pv. *campestris* during the interaction with the resistant and the susceptible cultivars. Differentially expressed proteins were identified, some of which were specifically expressed during the resistant interaction.

Materials and methods

Bacterial growth conditions in culture medium and *in planta*

Xanthomonas campestris pv. *campestris* 11078 (ATCC 33913), which had its complete genome sequenced (da Silva *et al.*, 2002), was cultured in the complex medium nutrient yeast glycerol (NYG) (Daniels *et al.*, 1984) at 28 °C. The infiltration and recovery of the bacterial cells from the resistant cultivar of *B. oleracea* (União) was performed as described by Mehta & Rosato (2003). Briefly, young leaves of cabbage were infiltrated with *X. campestris* pv. *campestris* ($A_{600\,nm} = 0.6$) and recovered at 1 and 2 days after inoculation (DAI). The infiltrated leaves were cut and submersed in *c.* 20 mL of distilled water in Petri dishes for 45 min. The bacterium was recovered from the suspension by centrifugation and used for protein extraction. The protein profile of the bacterium *in vivo* during the interaction with the resistant cultivar of *B. oleracea* at 1 DAI was compared with that of the bacterium recovered from the susceptible cultivar (Coração de boi) at 6 DAI. At these sampling points, the bacterium attained maximum growth in both interactions, as determined by the population dynamics study. Water-infiltrated leaves from the resistant cultivar were also analyzed in order to verify the possible presence of plant proteins. The same number of leaves used for bacterial protein recovery was collected for protein extraction.

Population dynamics was also performed by sampling disks of 6 mm diameter of infiltrated leaves at 0, 1, 2 and 3 DAI in the resistant plant. The disks were macerated in 1 mL sterile distilled water and diluted to appropriate concentrations. A total of 100 μL were plated onto NYG medium and incubated for a period of 2–3 days. The results were compared with those obtained for the susceptible plant at the sampling points of 0, 1, 2, 4 and 6 DAI (Andrade *et al.*, 2008).

Protein extraction and two-dimensional gel electrophoresis (2-DE)

Total protein was extracted from bacterial cells recovered from the resistant and the susceptible host plants and water-infiltrated leaves according to de Mot & Vanderleyden (1989). At least three different experiments for each condition were performed. An aliquot of 750 μL of extraction buffer (0.7 M sucrose, 0.5 M TrisHCl, 30 mM HCl, 50 mM EDTA, 0.1 M KCl and 40 mM dithiothreitol) and the same volume of buffer-saturated phenol were added to the sample. Proteins were precipitated with 0.1 M ammonium acetate in methanol, washed with 80% acetone, dried and stored at −20 °C. Protein quantification was performed using the Bradford Reagent (BioRad). Approximately 120 μg of protein was rehydrated with 2% (v/v) 3-[(3-chloramidopropyl) dimethylammonium]-1-propanesulfonate, 8 M urea, 7 mg dithiothreitol and 2% immobilized pH gradient (IPG) buffer. Isoelectric focusing was performed using 11-cm IPG strips with a pH range of 4–7 and a Multiphor II electrophoresis system (GE).

Second dimension analysis was performed in 10% acrylamide gels by sodium dodecyl sulfate polyacrylamide gel electrophoresis as described by Laemmli (1970) and at least five replications of each condition were performed. Protein spots were visualized after silver staining, according to Blum *et al.* (1987).

Image analysis

The 2D gel images (tiff files) obtained after silver staining were analyzed using the BIONUMERICS software v.4.5 (Applied Maths NV, Belgium). First, a calibration with a gray scale was performed to transform gray levels into OD values for each pixel (px) of the gel image. Spots were detected using the wizard detection method, proposed by the software, and the following parameters: 30 px for estimated spot size, 3 px for minimum spot size and a spot contrast enhancement of 75%. Automatically detected spots were manually checked and some of them were manually added or removed. Once the detection procedure was completed, the normalization step was performed to attribute a common spot identity to the same spots derived from different images using the reference gel construct and automatic matching options of the BIONUMERICS software. The overlapped images were based on landmark spots showing same pI and M_W in both gels, as well as molecular mass marker bands.

Trypsin digestion and MS analysis

Proteins were excised from the 2D gels stained with Coomassie brilliant blue and enzymatically digested into peptide fragments using Trypsin Sequencing Grade (Promega) according to Shevchenko *et al.* (1996). Aliquots of 1 μL of the digested protein were mixed with 1 μL of a saturated matrix solution of α-cyano-4-hydroxycinnamic acid, spotted onto matrix-assisted laser desorption/ionization (MALDI) target plates, and allowed to dry at room temperature. Monoisotopic masses of the molecular components ranging from *m*/*z* 600 to 6000 were determined by MS using an UltraFlex II

MALDI-TOF/TOF (Bruker Daltonics, Bilerica, MA) or a 4700 MALDI-TOF/TOF (Applied Biosystems, Framingham, MA) controlled using the manufacturer's softwares. All spectra were obtained in a positive reflector mode, using an accelerating voltage of around 20 kV for MS mode. Molecular ions displaying sufficient signals were submitted to MS/MS analyses, carried out in the positive mode precursor ion fragmentation at a laser frequency of 50 Hz. The MS/MS spectra were acquired in the reflector-positive mode after collision-induced dissociation or LIFT™ (Bruker Daltonics) fragmentation with external calibration. Resulting data were analyzed using FLEXANALYSIS 3.0 and the identification was performed by the MASCOT program (Matrix Science, London, UK) using the probability-based Mowse Score and a threshold of $P < 0.05$. Searches were performed against the NCBI database and only matches to *Xanthomonas* or plant species showing a significant score were considered reliable and therefore determined as positive identifications.

Results and discussion

The interaction between *X. campestris* pv. *campestris* and the susceptible and the resistant plants were compared in order to identify differentially expressed proteins. Initially, a population dynamics study was performed, and the results obtained revealed that in the resistant interaction, a population growth was observed only in the first 24 h after inoculation, starting from 3.5×10^6 CFU cm^{-2} and increasing to 7.1×10^7 CFU cm^{-2}. After this period, there was a 1000-fold reduction in the bacterial population, reaching 3.5×10^4 CFU cm^{-2} at 48 h after inoculation. At 72 h, no bacterial colonies were detected. On the other hand, the bacterial population in the inoculated susceptible cultivar decreased 100-fold in the first 24 h after inoculation and then increased, reaching 10^7 cells cm^{-2} at 6 DAI (Andrade *et al.*, 2008). These results show that the resistant plant blocks bacterial growth at 1 DAI.

The symptoms in the resistant and the susceptible plants were also quite different (Fig. 1). The susceptible plant showed black lesions with chlorotic margins, while the resistant plant presented light brown and dry necrotic lesions, indicating localized cell death.

The differential expression analysis of *X. campestris* pv. *campestris* in the interaction with the susceptible and the resistant plants was performed by 2-DE, and the 2D maps obtained revealed 152 and 178 protein spots, respectively, varying in molecular mass from 10 to 120 kDa and pI from 4 to 7 (Figs 2 and 3). The number of proteins found in both 2D maps seems to give a partial view of the entire *X. campestris* pv. *campestris* proteome, because a high number of proteins was not obtained. This is probably due to the limited amount of bacterial cells recovered from the plant. Indeed, a total of 120 μg of protein was loaded in the gel strips and a restricted pI range was used. In spite of the reduced number of proteins, several differentially expressed

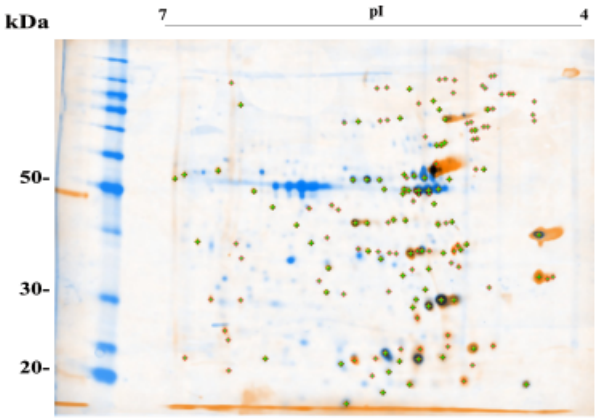

Fig. 2. Comparison of 2D gel images of *Xanthomonas campestris* pv. *campestris in vivo* susceptible (orange) and resistant (blue) interactions using BIONUMERICS software v.4.5. The 2D maps were overlapped in order to determine differentially expressed proteins. Protein spots observed under both conditions are marked by crosses. Green spots surrounded by orange borders indicate proteins exclusive of the susceptible interaction, and unmarked blue spots indicate proteins observed only in the resistant interaction.

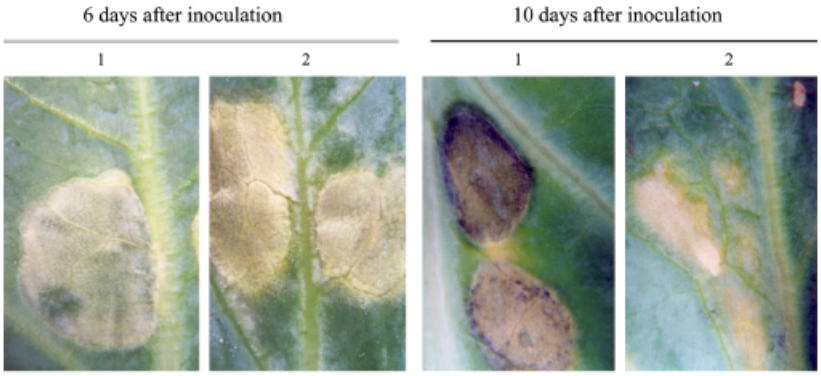

Fig. 1. Leaves from the susceptible (1) and the resistant (2) plants at 6 and 10 DAI with *Xanthomonas campestris* pv. *campestris*, visualized with a magnification of × 0.6. Black lesions with chlorotic margins can be observed in the susceptible plant as opposed to light brown and dry necrotic lesions in the resistant plant, which indicate localized cell death.

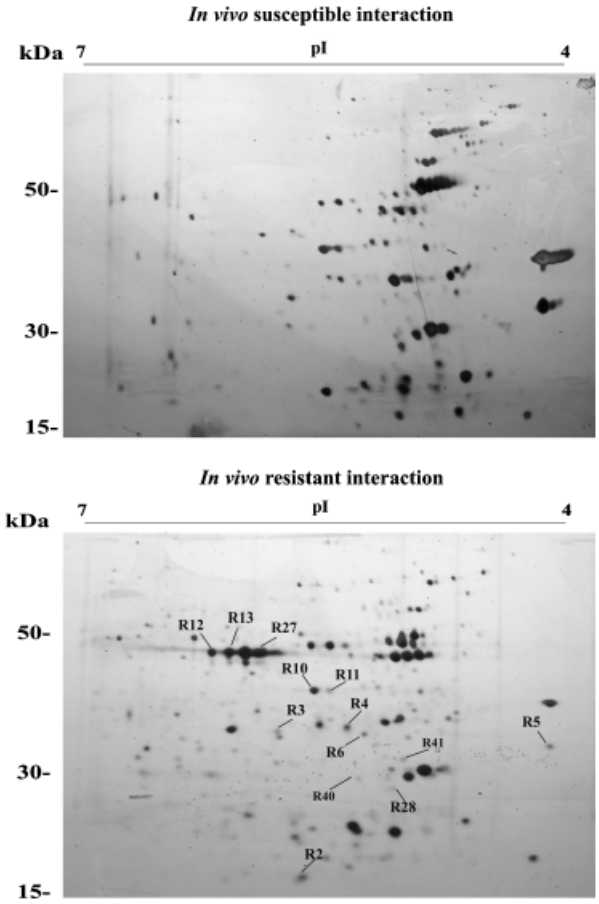

Fig. 3. 2-DE protein profile of *Xanthomonas campestris* pv. *campestris in vivo* in the interaction with the susceptible and the resistant cultivars, as indicated. Proteins (c. 120 μg) were visualized after silver staining and the highlighted spots indicate the proteins successfully identified by MS.

spots were observed when both 2D maps were overlapped and compared (Fig. 2). An intriguing result obtained was that a high number of exclusive proteins were observed in the *in vivo* resistant and susceptible profiles (105 and 79 proteins, respectively). A total of 73 common proteins observed represents *c.* 45% of the total proteins obtained in each 2D map. This result indicates that the host plant has a major influence in the protein expression pattern. In our earlier work (Andrade *et al.*, 2008), when the susceptible interaction was compared with culture medium conditions, the commonly expressed spots represented only 10% of the proteins observed in each 2D map. These results show that growth conditions are crucial and alter significantly the diversity of proteins observed in 2D maps, and that the sampling point is also a major factor that should be considered when comparing two different conditions. Although similar bacterial growth levels were used in this study to determine the sampling points in both interactions, the results indicate that the analysis at additional sampling points, perhaps at the log phase, could reveal a more comparable protein profile and therefore allow a more comprehensive and detailed analysis of the expression changes that occur in common protein sets.

In spite of the reduced number of commonly expressed proteins, expression-level analysis was performed, which revealed 49 spots showing differential expression above the threshold ratio of 1.5. A total of 20 differentially expressed proteins, not present in the plant 2D map, showing a higher intensity in Coomassie-stained gels were excised and analyzed by MS or MS/MS and positive identification was obtained for 11 proteins. In an attempt to identify a higher number of proteins, a total of 10 protein spots were also excised from silver-stained gels; however, only two of these spots were identified. The low percentage in the identification of silver-stained proteins is well known and represents a drawback when a low amount of protein sample is available for analysis, as is the case of the present study. In spite of these difficulties, exclusive and differentially expressed proteins could be identified and are discussed below.

Interestingly, a group of proteins of *c.* 50 kDa was observed only in the protein profile of the bacterium in the interaction with the resistant plant (Fig. 3). Because plant proteins were observed in our earlier work (Andrade *et al.*, 2008) when analyzing the susceptible interaction, a 2-DE was also performed using water-infiltrated leaves (control condition) from the resistant cultivar (Fig. 4). Indeed, several proteins were observed in the resistant plant 2D map and were therefore disregarded from the analysis, because they were also present in the bacterial protein profile. However, the group of 50-kDa proteins observed in the bacterium 2D map were not observed in the control condition, indicating that these proteins are specific to the interaction. Therefore, these protein spots, as well as other

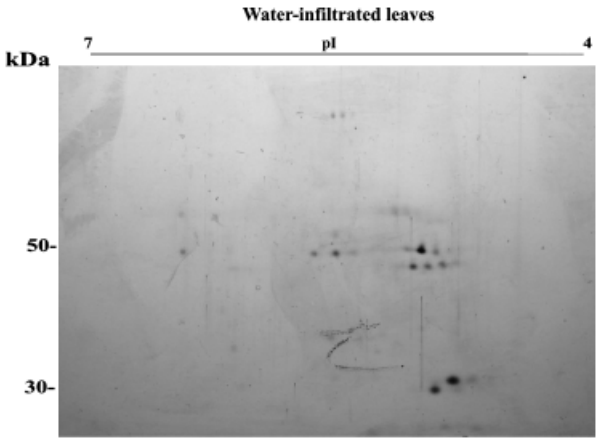

Fig. 4. 2-DE using water-infiltrated leaves of the resistant cabbage plant, showing some proteins also observed in the protein profile of the *Xanthomonas campestris* pv. *campestris in vivo* resistant interaction.

differentially expressed proteins, were then excised from the gel and analyzed by MS. The identified proteins are shown in Fig. 5 and Table 1.

Unexpectedly, three proteins (R12, R13 and R27), present only in the resistant interaction profile, were identified as ribulose bisphosphate carboxylase (Rubisco). It has been reported that photoinhibition may contribute to resistant responses in plants (Zou *et al.*, 2005). Tsunezuka *et al.* (2005) reported the downregulation of a fragmented Rubisco during lesion formation along several days in a resistant interaction in *Arabidopsis thaliana*. These authors suggested that degradation of Rubisco may have occurred as a result of oxidative stress. However, in this study, we observed the upregulation of proteins involved in photosynthesis in the resistant interaction, which included intact Rubisco subunits and an oxygen-evolving protein (spot R4). It is possible that a rapid cell disruption may have released these proteins in intact forms, allowing their detection in the 2D maps. Moreover, the sampling for protein extraction was performed at the beginning of infection (1 DAI) and, therefore, it is possible that there was no sufficient time for plant protein degradation to occur.

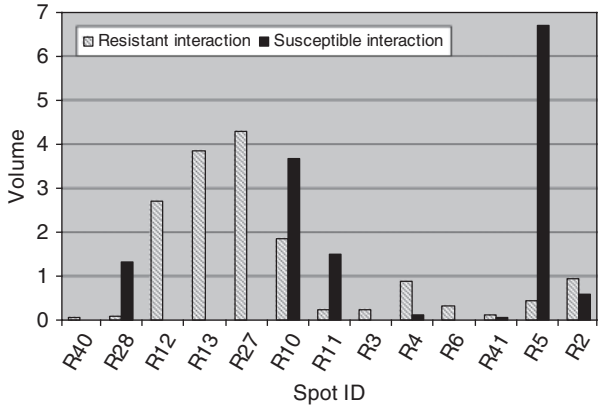

Fig. 5. Histogram representing expression levels of up- and downregulated proteins identified in the resistant interaction. The expression levels were determined using the corresponding spot volumes detected with BIONUMERICS software v.4.5.

Another photosynthetic protein identified in this study was the photosystem II (PSII) stability/assembly factor HCF136 (spot R11), which was downregulated during the resistant interaction (Fig. 5). HCF136 is important for the accurate assembly of PSII, which is a prerequisite for the proper functioning of the complex in the plant (Meurer *et al.*, 1998).

The role of proteins involved in photosynthesis in the plant–pathogen interaction appears to be unclear. While some photosynthetic proteins are upregulated, others are

Table 1. Proteins expressed during the resistant interaction identified by peptide mass fingerprinting or sequencing

Spot no.	Peptide sequence	Protein identification	Organism	Accession no.	Mascot score	M_r (gel)	pI (gel)	M_r (cal)	pI (cal)
R27	TFQGPPHGIQVE	Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit	*Oxandra xylopioides*	gb|AAW49419	72	50	5.9	50.7	6.1
R12	TFKGPPHGIQVER	Ribulose bisphosphate carboxylase	*Pandanus tectorius*	gi|343013	101	50	6.2	51.3	6.3
R13		Ribulose bisphosphate carboxylase large chain	*Brassica oleracea*	gi|1346967	88	50	6.1	52.9	5.8
R4	EVEYPGQVLR	Oxygen-evolving enhancer protein 2, chloroplast precursor	*Sinapis alba*	gi|131391	42	36.6	5.4	27.9	6.8
R11	SIPSAEEEDFNYR	High chlorophyll fluorescence 136 (HCF136)	*Arabidopsis thaliana*	gi|15237225	44	42.3	5.5	44.1	6.7
R3		Hypothetical protein	*Oryza sativa* Indica Group	gi|125551895	71	36	5.8	74.9	6.3
R6		EF-Tu	*X. campestris* pv. *campestris*	gi|21230362	79	35.7	5.3	43.1	5.4
R10	ALVENAGDIDAAAEWLR	Elongation factor Ts	*X. campestris* pv. *campestris*	gi|21112435	112	42	5.6	32.8	5.5
R41		Phosphohexose mutases	*X. campestris* pv. *campestris*	P0C7J2	100	32.4	5.1	48.8	5.2
R40		EF-Tu	*X. campestris* pv. *campestris*	Q4URC5	86	30.2	5.3	43.1	5.4
R2		Peptidyl-prolyl *cis–trans* isomerase	*X. campestris* pv. *campestris*	Q4UWH1	78	17.7	5.7	17.5	5.6
R5		Outer membrane protein	*X. campestris* pv. *campestris*	Q4URH9	139	34	4.2	39.3	4.5
R28		Membrane protein	*X. campestris* pv. *campestris*	Q8PD85	86	28.7	5.1	39.4	9.5

repressed. A more detailed analysis of the specific role of these proteins during plant–bacteria interaction needs to be conducted in order to correctly assign a biological function. A specific analysis of the differential expression of infected plant tissue may lead to more conclusive results regarding the expression of photosynthetic proteins during plant–pathogen interactions.

Among the bacterial proteins identified in this study was the *Xanthomonas* elongation factor Ts (spot R10), which was downregulated in the resistant interaction. It has been reported that this protein is induced in response to stress in *Escherichia coli* (Han *et al.*, 2007). This protein was also upregulated in the susceptible interaction when compared with water-infiltrated leaves (Andrade *et al.*, 2008).

Another protein identified (spots R6 and R40) was the elongation factor Tu (EF-Tu). This protein is one of the most abundant proteins in the bacterial cell and was recently identified as a general elicitor of defense responses and disease resistance in *Arabidopsis* (Kunze *et al.*, 2004). When analyzing the susceptible interaction in our previous study, several spots were identified as EF-Tu (Andrade *et al.*, 2008). However, spots R6 and R40 were observed only during the resistant interaction. These proteins probably represent isoforms, which may have a specific role in the interaction with the resistant host plant.

Membrane proteins (spots R5 and R28) were also identified as differentially expressed. Both proteins were highly upregulated in the susceptible interaction. Membrane proteins are of high importance in plant–pathogen interaction, because they may be part of secretion systems or may contribute for the attachment and aggregation of bacterial cells on the plant surface. It has been reported that outer membrane vesicles (OMVs), which contain outer membrane and periplasmic proteins (Dorward *et al.*, 1989; Mashburn & Whiteley, 2005), act as vehicles for the transportation of virulence-associated compounds into the host plant cells (Patrick *et al.*, 1996; Wai *et al.*, 2003). Sidhu *et al.* (2008) reported that OMVs released from *X. campestris* pv. *campestris* outer membrane during growth in culture media contain membrane- and virulence-associated proteins. The membrane proteins upregulated in the susceptible interaction of *X. campestris* pv. *campestris* in this study may play an important role in triggering the pathogenicity process in the host plant.

Overall, in this study, we have compared the protein profiles of *X. campestris* pv. *campestris* in the interaction with the susceptible and the resistant cultivars of *B. oleracea*. Differentially expressed proteins were detected during both interactions, and an interesting result obtained was the presence of different isoforms of the same protein in the resistant and the susceptible interactions. These results indicate that the same protein may play different roles depending on the type of interaction. A major difference observed was the pronounced reduction in bacterial cell counts in the resistant cultivar 48 h after inoculation and the presence of a group of 50-kDa proteins expressed exclusively under this condition. Unexpectedly, these proteins showed identity to plant photosynthetic proteins. The bacterial cell preparation used in this study involves cutting plant leaves instead of macerating in order to minimize plant tissue and protein contamination. However, plant proteins could still be detected in the bacterial 2D profile. It is possible that plant tissue degradation may have resulted in the release of these proteins, which are highly abundant in leaves. Another possibility is that, when bacterial cells are centrifuged for recovery, some plant debris may have also copelleted and, consequently, composed the protein extraction sample. Although some plant proteins were observed in the bacterial protein profile, most proteins were of bacterial origin. Further improvements in the bacterial cell recovery method may be performed in order to reduce the number of plant proteins detected. This study reports, for the first time, an *in vivo* global proteome analysis of *X. campestris* pv. *campestris* in the interaction with a resistant cultivar. A more detailed analysis of the plant proteome may reveal interesting proteins involved in the resistance to the bacterium. Further studies of the proteins identified may contribute to a better understanding of plant–pathogen interactions.

Acknowledgement

This work was supported by CNPq and Embrapa Recursos Genéticos e Biotecnologia.

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