Proteomic Analysis and Functional Validation of a *Brassica oleracea* Endochitinase Involved in Resistance to *Xanthomonas campestris*

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Black rot is a severe disease caused by the bacterium *Xanthomonas campestris* pv. *campestris* (Xcc), which can lead to substantial losses in cruciferous vegetable production worldwide. Although the use of resistant cultivars is the main strategy to control this disease, there are limited sources of resistance. In this study, we used the LC-MS/MS technique to analyze young cabbage leaves and chloroplast-enriched samples at 24 h after infection by Xcc, using both susceptible (Veloce) and resistant (Astrus) cultivars. A comparison between susceptible Xcc-inoculated plants and the control condition, as well as between resistant Xcc-inoculated plants with the control was performed and more than 300 differentially abundant proteins were identified in each comparison. The chloroplast enriched samples contributed with the identification of 600 additional protein species in the resistant interaction and 900 in the susceptible one, which were not detected in total leaf sample. We further determined the expression levels for 30 genes encoding the identified differential proteins by qRT-PCR. CHI-B4 like gene, encoding an endochitinase showing a high increased abundance in resistant Xcc-inoculated leaves, was selected for functional validation by overexpression in *Arabidopsis thaliana*. Compared to the wild type (Col-0), transgenic plants were highly resistant to Xcc indicating that CHI-B4 like gene could be an interesting candidate to be used in genetic breeding programs aiming at black rot resistance.

**Keywords:** LC-MS/MS, differential protein abundance, qRT-PCR, gene overexpression, plant–pathogen interaction

**INTRODUCTION**

Black rot, caused by the bacterium *X. campestris* pv. *campestris* (Xcc), is one of the most severe diseases that affects cruciferous crops. The use of resistant cultivars is the most efficient strategy to control black rot and therefore, resistance genes have been studied in *Brassica* genomes including the genome A (*B. rapa*), genome BC (*B. carinata*, originated from *B. nigra* x *B. oleracea*),...
and genome AC (B. napus, originated from B. rapa × B. oleracea). It is known that the characterized resistance genes can confer durable resistance to black rot in genomes A and B (Guo et al., 1991; Ignatov et al., 2000). However, there is limited information on resistance genes from genome C (B. oleracea), and there are no reports on resistance sources against black rot disease for this genome group (Camargo et al., 1995; Saba et al., 2016; Sharma et al., 2016).

Functional genomic and proteomic techniques have been important tools for exploring and understanding plant–pathogen interaction mechanisms. Proteomic studies can provide the link between gene expression and protein abundance and help identify key proteins involved in plant defense and resistance (Kamal et al., 2010a,b; Komatsu and Hossain, 2017). Although mass spectrometer sensitivity and software development have improved protein identification in the last years, there are still some limitations in the detection of low abundant proteins. One alternative to overcome this problem is the analysis of subcellular proteomes. This strategy can reduce sample complexity and provide the identification of a high amount of additional proteins contributing to a better understanding of the metabolic pathways involved (Stekhoven et al., 2014; Wang and Komatsu, 2016). Indeed, analyses of subcellular proteomes have been widely performed and presented a better picture of differential protein abundance under different stress conditions (Peltier et al., 2000; Uberegui et al., 2015). Chloroplasts have an important role in stress response and therefore the study of the chloroplast proteome can bring important contributions for the elucidation of plant defense, especially since this organelle participates actively in plant immune response (Audran et al., 2016).

In a previous study, Ribeiro et al. (2018) analyzed B. oleracea leaves inoculated with Xcc by 2-DE. Although differential protein spots were detected, the 2-DE technique is highly limited, particularly in the detection of low abundant proteins (Kamal et al., 2010a,b; Komatsu and Hossain, 2017). In this study, we performed bottom-up proteomics of inoculated leaves at the same time point (24 h after infiltration), in order to understand protein abundance at an early stage of infection. The total leaf proteome was further complemented with the analysis of chloroplast-enriched samples and the expression levels for 30 genes encoding the identified differential proteins were determined by qRT-PCR. Additionally, one protein was selected for overexpression in Arabidopsis thaliana to verify its involvement in resistance to Xcc.

**MATERIALS AND METHODS**

**Plant Material and Chloroplast Isolation**

In this work, two B. oleracea var. capitata cultivars, one moderately resistant (Astrus Plus – Chile/Seminis®) and one susceptible (Veloce – Brazil/Agristar®), as determined previously by our group (data not published), were used. A schematic figure of the experimental design is presented in the Supplementary Figure S1. The isolate of X. campestris pv. campestris (Xcc) Xcc51, obtained from Embrapa Hortaliças, Brasília, DF, Brazil, was used. Young plants (45 days after sowing) of both cultivars were inoculated with bacterial or saline (0.85% NaCl) solution, according to Santos et al. (2017). Leaves were harvested 24 hours after infiltration (hai), ground in liquid nitrogen and stored at −80°C. Three biological replicates, composed of five plants each, were analyzed. The same samples were used for chloroplast isolation using 5 g of ground material according to Kley et al. (2010), with modification in the percoll gradient. A 40%-80% percoll (Sigma-Aldrich) gradient was used.

**Protein Extraction and LC-MS/MS Analysis**

Leaves (0.3 g) and isolated chloroplasts (500 µL) were used for protein extraction according to Mot and Vanderleyden (1989), with modifications, as follows: for chloroplast protein extraction, we used a 1:2 extraction buffer (0.7 M sucrose, 0.5 M Tris-HCl, 50 mM EDTA, 0.1 M KCl, and 40 mM DTT):phenol proportion. Precipitated proteins were solubilized in urea/thiourea buffer (7 M urea; 2 M thiourea; 4% CHAPS, and 5 mM DTT) and quantified using the Bradford Reagent (Bio-Rad, Unite States). Approximately 150 µg of proteins from three biological replicates were loaded onto SDS-PAGE and allowed to migrate approximately 1 cm (Supplementary Figure S2) in a 12% resolving gel, as described by Valledor and Weckwerth (2014). Each gel lane containing one biological replicate was cut and submitted to in gel digestion using 5 µg of trypsin (Promega, Madison, WI, Unite States), according to Valledor and Weckwerth (2014). After the digestion procedure, the proteins were quantified using Quib™ fluorometer (Invitrogen), following the manufacturer’s instructions. Three biological and three technical replicates were analyzed by LC-MS/MS, totaling nine technical replicates. The peptide samples were desalted according to the protocol described by Rapplsieler et al. (2007) and suspended in 50 µL of 4% (v/v) acetonitrile (ACN) and 0.25% (v/v) formic acid.

A total of 2 µg of digested peptides were loaded into a one-dimensional (1D) nano-flow LC-MS/MS system (Thermo Scientific). Peptides were eluted using a monolithic C18 column Acclaim PepMap (Thermo Scientific) of 150 mm in length and 0.075 mm internal diameter. The gradient employed 0.1% formic acid in mobile phase A and 0.1% formic acid and 90% acetonitrile in mobile phase B during 180 min with a controlled flow rate of 400 nL/min from 5 to 35% phase B. The effluent from the nLC column was directly electrospayed into an Orbitrap Mass Spectrometer (LTQ-Orbitrap XL™ Hybrid Ion Trap, Thermo Scientific), operated in the positive ion mode and set to data-dependent acquisition.

Precursor peptides were detected in the mass range of 400–1,500 m/z and at a resolution of 120 K (at 200 m/z) with a target ion counting of 5 × 105. Tandem MS was performed by the isolation window of 1 atomic mass unit (amu), with CID (collision-induced dissociation) fragmentation in the quadrupole with a normalized collision energy of 35. The automatic gain control (AGC) was defined at 4 × 104 and the max injection time was of 50 ms. Only the 10 most intense precursors in the charge states of 2–6 were subjected to MS2. The dynamic exclusion duration was defined as 15 s with mass error tolerance.
and PEAKS with cycles of 3 s. The instrument was operated in max speed mode around 10 ppm. The instrument was operated in max speed mode with cycles of 3 s.

**Protein Identification and Quantification**

The raw data were processed using the software Progenesis QI (Nonlinear Dynamics, Waters, Durham, NC, United States) and PEAKS® 7 (Bioinformatics Solutions Inc., Waterloo, ON, Canada). A total of four pairwise comparisons (Xcc-inoculated vs. saline solution-inoculated) were performed (Supplementary Figure S3): (1) resistant Xcc-inoculated leaves compared to saline solution-inoculated leaves (LRI:LRC), (2) resistant Xcc-inoculated chloroplast compared to saline solution-inoculated chloroplast (ChRI:ChRC), (3) susceptible Xcc-inoculated leaves compared to saline solution-inoculated leaves (LSI:LSC), (4) susceptible Xcc-inoculated chloroplast compared to saline solution-inoculated chloroplast (ChSI:ChSC). The chromatograms from each comparison were automatically aligned and the alignment was manually revised for inconsistencies. Profile data from the MS scans were used to calculate the relative peptide abundance using the areas under the peaks of extracted ion chromatograms. Quantified features were median normalized and evaluated for statistical significance using ANOVA \( p \leq 0.05 \).

MS/MS files were exported as Mascot generic file (mgf) for peptide identification using PEAKS®7 (Bioinformatics Solutions Inc., Waterloo, ON, Canada) software (Zhang et al., 2012), and searched against the UniProt_Brassica oleracea database (taxonomy ID 3712) on February 2017. The analysis using PEAKS®7 was performed with the following parameters: peptide m/z tolerance ± 10 ppm; fragment ion m/z tolerance ± 0.5 Da; digestion with trypsin with two missed cleavages allowed; Cys carbamidomethylation as fixed modification and Met oxidation as variable modification. The search results were filtered with FDR < 1%. The SPIDER tool within the software PEAKS®7 was used to find homologous peptides presenting a single amino acid substitution in the database (Han et al., 2005). The data generated was deposited in the MassIVE repository (DOI: 10.25345/C5KG6W).

**qRT-PCR Analysis**

Total RNA was extracted from the same leaf samples (0.1 g) used for protein analysis by the TRizol® Reagent method (Invitrogen™), following the manufacturer’s instructions. RNA samples were quantified using a NanoDrop™ 200 spectrophotometer (Thermo Scientific) and the integrity of the RNA was observed in 1% agarose gel. RNA was treated with Turbo™ DNase (Applied Biosystems/Ambion) and cDNA synthesis was performed using 2 µg of total RNA and the GoScript™ Reverse Transcription System (Promega), following the manufacturer’s instructions. A total of 30 genes encoding the differentially abundant proteins identified were selected for qRT-PCR (Supplementary Table S1). SAND (SAND family protein), TBPI (TATA-box-binding protein 1), TUB6 (Tubulin beta-6), and UBQ1 (Ubiquitin-60S ribosomal protein L40) were used as reference genes. All primers used were designed using Primer3Plus program (Untergasser et al., 2007). qRT-PCR was performed using three biological and three technical replicates, as described by Santos et al. (2017). The analysis was performed in a thermal cycler 7300 Real-Time PCR System (Applied Biosystems). To verify the absence of genomic DNA in the samples, qRT-PCR was performed using RNA as template. For stability evaluation of the reference genes, the geNorm algorithm (Etschmann et al., 2006) was used and the expression analysis was performed with the REST software (Pfaffl et al., 2002).

**Overexpression of BoCHI-B4 Like Gene in Arabidopsis thaliana**

The gene BoCHI-B4 like (GAQY01039586) encoding the basic endochitinase CHB4-like protein (A0A0D3BPL2) was selected for functional validation. The binary vector pBin61 that carries a transcription cassette with the CaMV 35S promoter and terminator, and the kanamycin resistance gene as selection marker was used (Bendahmane et al., 2002). The BoCHI-B4 like gene (BoCHI4) was synthesized and cloned into the pBIN61 vector by Epoch Life Science Inc. (Missouri City, TX, United States) to generate the construct pBIN61: BoCHI4, which was used to transform A. thaliana (Col-0), mediated by Agrobacterium tumefaciens (strain GV3130) using the floral dip method (Bent and Clough, 1998). Approximately 0.5 g of seeds of the transformed plants were sterilized and distributed in MS culture medium supplemented with kanamycin (50 mg L\(^{-1}\)) resulting in the selection of 15 pBIN61:BoCHI4 primary transformants. The parental lines and T2-generation were germinated in MS medium containing kanamycin and transferred to substrate and maintained in a growth chamber under a 12 h light:12 h dark photoperiod at 22°C. To confirm transformation, leaves were harvested for DNA extraction, followed by PCR amplification and sequencing using specific primers. DNA of non-transformed plants (wild type Col-0) was used as control.

**Molecular and Phenotypical Characterization of Arabidopsis Transgenic Lines**

Leaves of T2 homozygous events were harvested for DNA and RNA extraction for Southern blot and qRT-PCR analysis. For Southern blot, DNA extracted from T2-generation plants (10 µg) was digested with XbaI and analyzed using standard procedure (Romano and Vianna, 2015). The RNA preparation and qRT-PCR analysis were performed as described above. The A. thaliana reference genes ACT2 (Actin 2) and EF-1a (elongation factor-1a) were used (Supplementary Table S1). The effect of gene overexpression was confirmed by spraying bacterial solution (Xcc51 OD\(_{600}\) = 0.1) followed by disease development scoring from 1 to 5 days post inoculation (dpi) using a disease index ranging from 0 (no symptom, considered highly resistant) to 4 (full leaf necrosis, classified as highly susceptible), based on (Meyer et al., 2005). A total of five events was evaluated and 15 plants from each event were analyzed, as well as 15 wild type Col-0 plants, used as the control.
RESULTS AND DISCUSSION
Proteomic Profile and Gene Expression Analysis of Brassica Leaves and Chloroplast Enriched Samples

In this study, two conditions were compared for the identification of differentially abundant proteins: Xcc-inoculated and saline solution-inoculated (control condition) leaf and chloroplast from resistant and susceptible cultivars (Astrus and Veloce), resulting in four comparisons (Supplementary Figure S3). As observed in our previous studies (data not published), Astrus was moderately resistant to Xcc51 and Veloce was highly susceptible (Figure 1). The LC-MS/MS data analysis resulted in more than 30,000 peptide sequences, corresponding to more than 1,000 protein species (Supplementary Table S2). Since several of the matches corresponded to uncharacterized proteins in the Uniprot database, a second analysis was performed using Blas2GO software to identify the proteins and infer the gene ontology (GO) for biological process. The leaf proteome of the resistant cultivar inoculated with Xcc (LRI) and in the control condition (LRC) revealed a total of 1,424 proteins, while the leaf samples of the susceptible cultivar Veloce inoculated with Xcc (LSI) and in the control condition (LSC) revealed a total of 1,395 proteins (Supplementary Figure S3).

Chloroplast enriched samples from the resistant cultivar inoculated with Xcc (ChlRI) and in the control condition (ChlRC) as well as from the susceptible cultivar Veloce inoculated with Xcc (ChlSI) and in the control condition (ChlSC) were also analyzed (Supplementary Figure S3). The proteins identified in the leaf and chloroplast enriched samples from each cultivar were merged into a single table (Supplementary Table S2) for discussion, totaling 2,086 proteins in the resistant interaction, referred to as RI:RC and 2037 in the susceptible interaction, referred to as SI:SC (Supplementary Figure S3). Proteins with the same name were aligned for sequence comparison (protein sequence alignment) using ClustalOmega2 and when differences in the sequences were observed they were considered as different protein species. The results showed that the proteome from chloroplast samples contributed with 662 additional proteins in the resistant plants (ChlRI and ChlRC) and 642 in the susceptible (ChlSI and ChlSC) (Supplementary Figure S3), which were not detected in leaf samples. Moreover, a total of 338 differentially abundant proteins were identified in the resistant interaction (RI:RC), 200 of which were obtained from chloroplast enriched samples (Xcc-inoculated compared to the control). The susceptible interaction (SI:SC) revealed 361 differential proteins out of which 175 were identified in chloroplasts (Supplementary Table S3). These results emphasize that the analysis of subproteomes can contribute significantly for the identification of additional proteins (Rolland et al., 2012; Bayer et al., 2015), especially those present in lower abundance (Kim and Kang, 2008).

In this work, we also analyzed the gene expression levels by qRT-PCR of 30 selected genes encoding the differential proteins identified (Figure 2 and Table 1), based on biological process (defense-related), fold-change ($\geq 1.5$ increased or decreased in both cultivars) and previous studies (Villeth et al., 2016; Ribeiro et al., 2018). As expected, the expression levels of many mRNAs did not correlate with protein abundance and different clusters could be observed in the heatmap generated to compare these levels (Figure 2). In the resistant cultivar, among the 14 proteins showing increased abundance (statistically validated), 5 corresponding genes showed upregulation (BoAMCA4; BoANNA2; BoCHB4; BoBGP1; BoFPSI), and among the 4 proteins showing decreased abundance, 1 corresponding gene showed downregulation (BoPER32) and the others were not statistically significant. Similar results were obtained for the susceptible cultivar: 10 proteins showed increased abundance, out of which 4 corresponding genes were upregulated, while 8 proteins showed decreased abundance and 2 corresponding genes showed downregulation (BoENH1 and BoPRX2F).

The differences observed between protein abundance and gene expression levels has been widely reported and may be explained by the regulatory processes that can occur after mRNA transcription, including post-transcriptional, translational, post-translational and protein degradation regulation mechanisms, as well as half-life of RNA and of the corresponding proteins (de Sousa Abreu et al., 2009; Lee et al., 2011; Vogel and Marcotte, 2012).

Xcc-Responsive Proteins in the Resistant and Susceptible Interaction

The proteome analysis of the resistant cultivar (RI:RC) revealed 338 differentially abundant proteins (215 increased and 123 decreased) while in the susceptible cultivar comparison (SI:SC) 361 differential proteins (225 increased and 136 decreased) were detected. The GO analysis revealed the same over-represented GO terms in both resistant and susceptible interactions (Supplementary Table S3), including cell metabolism, protein biosynthesis, processing and degradation, photosynthesis, disease/defense response and uncharacterized proteins (proteins with no GO information).

A higher number of energy metabolism proteins (Supplementary Table S3) were identified in the susceptible cultivar, most of which showed decreased abundance. It is

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2https://www.ebi.ac.uk/Tools/msa/clustalo
FIGURE 2 | Heatmap showing the correlation between protein abundance (PA) and gene expression (GE) levels in the resistant (A) and in the susceptible interaction (B). (C) Gene expression of 30 genes in leaves of B. oleracea 24 h after inoculation with X. campestris pv. campestris (Xcc) compared to the control condition. The symbol * indicates statistically significant differential expression ($p \leq 0.05$). The full information of genes and gene products are presented in Table 1. Bo, Brassica oleracea gene name homologous to A. thaliana.
| Differential genes analyzed by qRT-PCR and included in the model | Gene product (full name) | UniProt Accession # | Protein Fold change | Gene Fold change (log2) | Gene ontology (biological process) |
|---|---|---|---|---|---|
| BoAKR2A | Ankyrin repeat domain-containing protein 2-like | AKR2 | A0A0D3BK51 | ni | -2 | -0.37 | -0.003 | Protein targeting to chloroplast |
| BoAMC4 | Metacaspase-4 | MC4 | A0A0D3D1T5 | 2 | 2 | 2.88* | 1.172* | Positive regulation of programmed cell death; protein autoprocessing |
| BoANN2 | Annexin | ANN2 | I3Y171 | 2 | ni | 3.80* | 2.357* | Calcium ion transmembrane transport; response to oxidative stress |
| BoAPYS | Apyrase 5 | APYS | A0A0D3CA22 | nd | -10 | 0.73 | 0.179 | None predicted |
| At5g02240 | Uncharacterized protein At5g02240 | At5g02240 | A0A0D3BD2 | nd | 3 | -0.31 | 0.897* | Response to abscisic acid |
| BoATPC1 | ATP synthase gamma chain 1, chloroplastic | ATPG1 | A0A0D3E873 | nd | -11 | -0.23 | -0.188 | ATP synthesis coupled proton transport |
| BoBAG7 | BAG family molecular chaperone regulator 7-like | BAG7 | A0A0D3A4W0 | ni | -81 | 1.21 | 0.452 | Cellular response to unfolded protein; protein folding; cellular response to heat |
| BoCHB4 | Basic endochitinase CHB4-like | CHB4 | A0A0D3BPL2 | 6 | ni | 9.17* | 4.495* | Cell wall macromolecule catabolic process; chitin catabolic process; response to virus; systemic acquired resistance |
| BoCLPP | ATP-dependent Clp protease proteolytic subunit | CLPP | A0A0D3A7F3 | ni | -2 | 1.18 | -0.385 | Protein quality control for misfolded or incompletely synthesized proteins |
| BoDEGP8 | Protease do-like 8, chloroplastic | DEGP8 | A0A0D3BW4 | 3 | ni | 0.35 | 6.457* | Photosystem II repair |
| BoDTX | Protein detoxification | DTX | A0A0D3BJ77 | ni | 2 | 0.54 | 0.268 | Abscisic acid transport; drug transmembrane transport; regulation of response to water deprivation |
| BoENH1 | Rubredoxin_like, 1 | ENNH1 | A0A0D3A4G1 | nd | -2 | -1.58* | -0.639* | None predicted |
| BoEF1-3 | Eukaryotic peptide chain release factor subunit 1–3 | eEF1-3 | A0A0D3DQ3 | 3 | nd | 1.05 | 0.317 | Cytoplasmic translational termination; regulation of growth; translational termination |
| BoESP | Epithiospecifier-like | ESP | A0A0D3CQU9 | nd | -2 | 4.56* | 1.607* | Defense response to bacterium; catabolic process; nitrate biosynthetic process; response to jasmonic acid |
| BoFSD1 | Superoxide dismutase | SOD | F8U7Z7 | 3 | nd | 0.71* | -0.017 | Cellular response to oxidative stress; defense response to bacterium; cellular response to salt stress; cellular response to UV-B |
| BoGASA1 | Gibberellin-regulated protein 1 | GAST1 | A0A0D3D0Y9 | 2 | nd | 0.01 | -1.905* | Response to abscisic acid; response to brassinosteroid; response to gibberellin |
| BoIF-2 | Translation initiation factor IF-2 | IF2 | A0A0D3CZ7 | -2 | nd | -1.30 | 0.110 | Translational initiation; translation; nucleotide binding |
| BoLLP | Lectin-like protein A8g16530 | LLP | A0A0D3CJY3 | 3 | ni | -6.51* | 3.470* | Response to chitin; response to oomycetes |

(Continued)
### TABLE 1 | Continued

| Gene\(^1\) | Gene product (full name)                      | Protein ([SN])\(^2\) | UniProt Accession # | Protein Fold change | Gene Fold change (log\(_{2}\)) | Gene ontology (biological process)\(^3\) |
|------------|-----------------------------------------------|----------------------|---------------------|---------------------|--------------------------------|------------------------------------------|
| **Differential genes analyzed by qRT-PCR and included in the model** | | | | | | |
| BoMLP31    | MLP-like protein 31                          | MLP31                | A0A0D3APR4          | −4                  | −0.83                          | −0.860*                                  | Defense response                        |
| BoPER32    | Peroxidase 32                                 | PEROX32              | A0A0D3E2V6          | −2                  | 1.92*                          | 0.239                                   | Response to oxidative stress; hydrogen peroxide catabolic process; response to cytokinin |
| BoPIP1-1   | Aquaporin PIP1b1                               | PIP1b1               | Q9FUL1              | ni                  | 2                              | −0.46                                   | Water transport; response to oxidative stress; response to water deprivation |
| BoPPD4     | pspB domain-containing protein 4, chloroplastic | PSBP4                | A0A0D3D1B1\(^4\)   | nd                  | 9                              | −0.64                                   | Photosynthesis                          |
| BoPRX2F    | Peroxiredoxin IIF, mitochondrial              | PRXII                | A0A0D3CMF0\(^4\)   | −2                  | 2                              | −0.33                                   | Cell redox homeostasis; response to cadmium ion; response to oxidative stress |
| BoPSBE     | Cytochrome b559 subunit alpha                 | PSBE                 | A0A0H3Y313\(^4\)   | nd                  | −2                             | −0.44                                   | Photosynthetic electron transport chain |
| BoPSBD     | Photosystem II D2 protein                     | PSI1 D2              | A0A191SEU8          | nd                  | 2                              | −0.13                                   | Photosynthetic electron transport in photosystem II; protein-chromophore linkage |
| BoRFK1     | LRR receptor-like serine/threonine-protein kinase A1tg29720 | LRR-RLK              | A0A0D3CSX5          | 2                   | nd                             | −0.71                                   | Protein autophosphorylation; regulation of innate immune response; jasmonic acid and ethylene-dependent systemic resistance |
| BoRG1      | UDP-arabinopyranose mutase 1-like             | UAM                  | A0A0D3B9D8          | 9                   | ni                             | 2.20*                                   | Plant-type cell wall organization or biogenesis |
| BoTL17     | Thylakoid luminal 17.4 kDa protein, chloroplastic | P17.4               | A0A0D3AP2\(^4\)   | 3                   | 6                              | −0.69                                   | Protein binding                         |
| BoYCF54    | Uncharacterized protein Ycf54                 | YCF54                | A0A0D3CB6\(^4\)    | 3                   | 3                              | −0.38                                   | None predicted                          |
| BoUSPA     | Universal stress protein YxIE-like            | YXIE                 | A0A0D3OTQ8          | 2                   | 2                              | 0.73                                    | None predicted                          |
| **Additional proteins included in the model** | | | | | | |
| BoTRXM     | Thioredoxin M chloroplastic                   | TRXM                 | A0A0D3DZS3\(^4\)   | 2                   | 2                              | Cell redox homeostasis; glycerol ether metabolic process; regulation of carbohydrate metabolic process |
| BoPRXQ     | Peroxiredoxin Q, chloroplastic isoform x2     | PRXQ                 | A0A0D3FBQ6\(^4\)   | 2                   | nd                             | Cell redox homeostasis                 |
| BoPRX      | Peroxiredoxin- Chloroplastic                 | PRX                  | A0A0D3BDY5\(^4\)   | 2                   | nd                             | Cell redox homeostasis                 |
| 106329510  | Pectinesterase                               | PEM17                | A0A0D3B6U2\(^4\)   | 4                   | 2                              | Cell wall modification; pectin catabolic process |
| BoGSTU5    | Glutathione S-transferase U5                  | GSTU5                | A0A0D3B771          | 2                   | nd                             | Response to oxidative stress; response to toxic substance; toxin catabolic process |

(Continued)
| Gene                  | Gene product (full name)            | Protein (SN) | UniProt Accession # | Protein Fold change | Gene ontology (biological process) |
|-----------------------|-------------------------------------|--------------|---------------------|---------------------|-----------------------------------|
| Additional proteins included in the model | | | | | |
| BoGSTU19              | Glutathione S-transferase U19       | GSTU19       | A0A0D0CVZ5          | 2                   | R S                               |
| BoBoAIG2              | Aig2 protein                        | AIG2         | A0A0D0BZV6          | 2                   | Response to bacterium             |
| 106337169             | Ferredoxin                          | FDX          | A0A0D0BV84          | 2                   | Transport; electron transport     |
| BoLFNR                | Ferredoxin–Nadp leaf isozyme 1     | FNR          | A0A0D0E2R4          | 1.5                 | ni                                |
| BoLFNR2               | Ferredoxin–Nadp leaf isozyme 2     | FNR2         | A0A0D0DQ24          | 2                   | Defense response to; defense response to fungus, incompatible interaction; photosynthesis; response to cytokinin |
| BoGF14 BoKAPPA        | 14-3-3 GF14 kappa isoform X1        | GF14 kappa   | A0A0D0BET0          | 2                   | Regulation of metabolic; response to freezing |
| BoAKR4C8              | Aldo-keto reductase family 4        | AKR4C8       | A0A0D0BR44          | 2                   | Oxidation-reduction process; response to cadmium ion; response to toxic substance; response to cold; response to salt stress |
| BoPBH2                | Prohibitin 2, mitochondrial-like    | mtPBH2       | A0A0D0C7E7          | 2                   | Mitochondrion organization; cell division; defense response to bacterium; negative regulation of cell division; response to auxin |
| BoVDAC4               | Mitochondrial outer membrane porin 4| mtVDAC4      | A0A0D0B2Z9          | 2                   | Regulation of growth; response to bacterium |
| BoMDH                 | Malate dehydrogenase mitochondrial | mtMDH        | A0A0D0CQE1          | 3                   | Carbohydrate metabolic process; malate metabolic process; tricarboxylic acid cycle |
| BoMDH                 | Malate dehydrogenase, chloroplastic | chlMDH       | A0A0D0CGY3          | 2                   | Carbohydrate metabolic process; malate metabolic process; tricarboxylic acid cycle |
| 106341843             | Glucan endo-1,3-beta-glucosidase-like | BG           | A0A0D0BXB6          | 7                   | Carbohydrate metabolic process |
| 106300472             | Glucan endo-1,3-beta-glucosidase-like (beta-1,3-glucanase) | BG_ppap    | A0A0D0CTF1          | 2                   | Carbohydrate metabolic process; cell communication |
| BoGAPDH               | Glyceraldehyde-3-phosphate dehydrogenase, chloroplastic | chlGAPDH  | A0A0D0DXN8          | 2                   | Glucose metabolic process |
| BoPAP 1               | Plastid-lipid-associated 1, chloroplastic | chlPAP 1 | A0A0D0EBB6          | 2                   | Photoinhibition; response to abscisic acid; response to cold |
| BoPAP 2               | Plastid-lipid-associated 2         | PAP 2        | A0A0D0B8J8          | 2                   | None predicted                    |
| BoPAP 3               | Plastid-lipid-associated 3         | PAP 3        | A0A0D0B8J8          | 2                   | None predicted                    |
| BoUSPA                | Universal stress A                 | USP-A        | A0A0D0C0N1          | 2                   | None predicted                    |
| BoPEPR1               | Leucine-rich repeat receptor kinase Pepr1 | PEP1       | A0A0D0D099          | 3                   | Immune response; innate immune response; response to jasmonic acid; response to wounding |

(Continued)
**TABLE 1 | Continued**

| Gene | Gene product (full name) | UniProt Accession # | Protein Fold change | Gene ontology (biological process) |
|------|--------------------------|---------------------|---------------------|-----------------------------------|
| NA   | Leucine-rich repeat receptor-like serine | A0A008BD14 | 2 | Protein autophosphorylation; regulation of innate immune response; jasmonic acid and ethylene-dependent systemic resistance |
|      | threonine-kinase A3g14840 | LRR-RLK | ni | |
| BoPT12 | PT11-like tyrosine-protein kinase 2 | A0A008B4A1 | 1.5 | Defense response; protein phosphorylation |
| BoGAPC | Glyceroldehyde-3-phosphate dehydrogenase | A0A002ZP69 | −2 | Glucose metabolic process |
|      | | | | |
| 106335373 | Malate dehydrogenase [NADP] | A0A008B2U9 | −2 | Carbohydrate metabolic process; malate metabolic process |
|      | chloroplastic-like | chMDH | ni | |
| BoGPX1 | Glutathione peroxidase mitochondrial | mtGPX | −2 | Response to oxidative stress |
| BoDHAR1 | Glutathione S-transferase DHAR1, mitochondrial-like | mtDHAR | −2 | Cellular response to hydrogen peroxide; defense response; positive regulation of salicylic acid mediated signaling pathway; response to jasmonic acid |
| BoPER3 | Peroxidase 3-like | PEROX3 | −2 | Hydrogen peroxide catabolic process; response to oxidative stress |
| BoPER32 | Peroxidase 32 | PER32 | −2 | Hydrogen peroxide catabolic process; response to cytokinin; response to oxidative stress |
| BoLHC65 | Chlorophyll a-b binding protein CP26, chloroplastic | OP26 | −4 | Non-photochemical quenching; photosynthesis, light harvesting; photosystem II assembly; protein-chromophore linkage |
| BoLHC64.2 | Chlorophyll a-b binding protein CP29.2, chloroplastic | OP29.2 | −2 | Photosynthesis, light harvesting; protein-chromophore linkage; response to blue light; response to cytokinin |
| NA | Chlorophyll a-b binding protein CP43, chloroplastic | OP43 | −2 | Photosynthetic electron transport in photosystem II; protein-chromophore linkage |
| BoRPN | 26S proteasome non-ATPase regulatory subunit 5 | RPN5 | −2 | Proteasome assembly; translation |
| BoRPT3 | 26S protease regulatory subunit 6B homolog | 26Sp6B | 2 | Protein catabolic process |
| BoUBC36 | Ubiquitin-conjugating enzyme E2 36 | UBC36 | −2 | Postreplication repair; protein K63-linked ubiquitination |
| BoUBC7 | Ubiquitin-conjugating enzyme E2 7 | UBC7 | −2 | Protein ubiquitination |

1. Brassica oleracea (Bo) gene name based on the homolog in Arabidopsis thaliana. “N/A” refers to non-annotated. 2. Short name of gene product. nd, non-differential protein (fold change < 1.5); ni, non-identified proteins in one of the cultivars. The positive numbers indicate gene fold change up-regulated or increased abundance of gene product and the negative down-regulated or decreased abundance. Log2 results presentation of fold change values for gene relative expression evaluated by qRT-PCR. R, resistant; S, susceptible. 3. Gene ontology; summing the principal biological process terms showed in database; mt, mitochondrial; chl, chloroplastic, prefix used here for differentiation of gene product localization. 4. Gene product identified in enrichment chloroplast simple. “Differential” gene expression statistically validated (p ≤ 0.05).
known that there is intense activity of the main glycolytic pathways during plant–pathogen interaction and alterations in sugar metabolism in the host plant can be crucial for pathogen control, since both organisms compete for nutrients (reviewed by Kanwar and Jha, 2019). It is important to highlight that most of the differential proteins related to energy metabolism were detected only in chloroplast enriched samples, which reinforces the importance of analyzing organelle enriched samples to get a better picture of the plant–pathogen interaction. It is known that photosynthesis is severely affected during biotic and abiotic stresses since resistance has an energy cost. Although the molecular participation of chloroplast in plant immunity is not clear, it has been shown that chloroplasts can have a crucial role in the plant basal immune system that involves PAMPs signaling, Ca²⁺ signaling pathways, as well as salicylic and jasmonic acid (JA) production (Grant and Jones, 2009; Padmanabhan and Dinesh-Kumar, 2010; Nomura et al., 2012).

Energy Metabolism Proteins

Three malate dehydrogenase mitochondrial proteins (A0A0D3CQX1; A0A0D3CQX2) showed increased abundance in both cultivars, however, one of them (A0A0D3BMU9) showed increased abundance in the resistant cultivar (4-fold) and decreased abundance in the susceptible one (14-fold), when compared to the control condition. Malate is implicated in many plant metabolic processes, including TCA cycle, Calvin cycle, and in pH regulation and ion transport in roots. Malate dehydrogenase, an important malate metabolizing enzyme, has been associated with plant defense, suggesting that the increased abundance of this enzyme can provide resources for biosynthesis of defense compounds (Rhodes et al., 1968; Walter et al., 1988; Casati et al., 1999). In a previous study, one MDH1 showed increased abundance in brassica–Xcc resistant interaction and was associated with the activation of photosynthetic metabolism (Villeth et al., 2016). Other metabolism proteins such as fructose-1,6-bisphosphate, cytosolic EC 3.1.3.11 (A0A0D3BSL1), basic endochitinase CHI-B4-like, EC 3.2.1.14 (A0A0D3B6J8; A0A0D3BPL2) and UDP-arabinopyranose mutase 1-like (A0A0D3BD98), were increased in RI (9-, 12-, 9- and 6-fold, respectively) and the first two (A0A0D3BSL1 and A0A0D3B6J8) were decreased in SI (28- and 12-fold, respectively), while UDP-arabinopyranose level was unchanged in SI. In the analysis of gene expression (Figure 2 and Table 1), BoCHI-4 like and BoRGP1 were upregulated, with a higher expression in RI:RC (578 and fivefold, respectively) when compared to SI:SC (23- and 4-fold, respectively), suggesting that these proteins, besides being involved in energy metabolism, can have an important role in plant defense. Based on the proteomic and qRT-PCR results, the basic endochitinase BoCHI-B4-like gene (GAQY1039586.1) was selected for overexpression in the model plant A. thaliana for functional validation.

The metabolic pathways role in defense response process is not well understood, however, our results were consistent with other studies, which suggest that the positive regulation of metabolism can initiate a signaling cascade in the signal transduction pathway, leading to a defense response (Rojas et al., 2014). On the other hand, the pathogen can acquire metabolites from the host cell and the plant can respond to prevent the loss of metabolites by increasing the uptake of monosaccharides, limiting the available extracellular sugar for bacteria. This could be a strategical antimicrobial response, since this competitive reaction can lead to the restriction of the delivery of virulence factors (Vogel and Marcotte, 2012; Couto and Zipfel, 2016; Yamada et al., 2016). Indeed, in Brassica and Arabidopsis it has been demonstrated that sugar transporters, such as SWEET transporters that mediate sugar export are positively regulated upon pathogen infection (Chen et al., 2010, 2012; Jian et al., 2016), which could indicate co-evolution for nutrient competition during plant–pathogen interaction (Chen et al., 2010). Studies have suggested that Xanthomonas effectors can recognize SWEET proteins from the plant and induce sugar export from the cell to be used as carbon source for bacterial growth (Cohn et al., 2014; Huang et al., 2016).

Proteins Involved in Photosynthesis and Protein Biosynthesis, Processing and Degradation

As expected, several photosynthesis-related proteins were differentially abundant in both interactions, such as photosystem II CP43 reaction center protein, chlorophyll a-b binding protein CP29.2 and CP26, Ribulose bisphosphate carboxylase (Supplementary Table S3). Most proteins related to photosynthesis in the resistant interaction showed decreased abundance (18%), which is consistent with previous results obtained by Ribeiro et al. (2018), when analyzing the chloroplast enriched samples and observed that several of the proteins with increased abundance were detected in chloroplast samples (Supplementary Table S3), which once again reinforce the importance of subproteome analysis to better understand the global protein interaction profile.

In this study, a clear imbalance in metabolic and photosynthetic processes in both cultivars could be observed, however, it is possible that the resistant plant may have a higher recovery capacity than the susceptible plant, since homeostasis and repair proteins were more abundant in the resistant interaction than in the susceptible. It is known that the impaired metabolic capacity can directly influence the functioning of the photosynthetic apparatus (Raven et al., 2007), correlating metabolic alterations with response to pathogens.

Another over-represented GO term observed in this study was protein biosynthesis, protein processing and degradation (folding, assembly, fate and degradation). Proteins mainly involved in transcription, translation, post-translational and transduction processes were observed with increased abundance in both interactions, including several ribosomal proteins (30S, 40S, and 50S in RI:RC; 50S and 60S in SI:SC). It is noteworthy that the BAG (Bcl-2 associated athanogene) family molecular chaperone regulator 7-like (A0A0D3A4W0) showed a pronounced decreased abundance (81-fold) in SI compared to the control, and in resistant plants this protein was not
detected. qRT-PCR results showed an upregulation trend in RI and downregulation trend in SI (Figure 2). These results suggest post-transcriptional or post-translational regulation events, since mRNA and protein levels were highly distinct. The BAG7 protein is a member of Class III of BAG family proteins, which is composed by eight proteins encoded by highly conserved genes, widely distributed in living organisms (Weissbach et al., 1994; Takayama et al., 1995). In plants, BAG proteins have been considered multifunctional and known to regulate the cytoprotective process during abiotic and biotic stresses (Doukhchina et al., 2006). Li et al. (2016) identified proteins of this family that confer resistance in Arabidopsis against the fungal pathogen Botrytis cinerea, showing evidence of the participation of BAG proteins in innate immunity processes.

**Disease/Defense Response Proteins**

Among the disease/defense response proteins identified in our study, most were increased in both cultivars and were involved with oxidative stress (Supplementary Figure S4). However, a higher number of pathogen-related proteins associated with plant responses showed increased abundance in the resistant cultivar (16%), whereas only a few (5%) were increased in the susceptible cultivar. The increased defense proteins identified in both cultivars were annexin (I3Y171), AIG2 (A0A0D3BZV5), ferredoxin (A0A0D3BV84), ferredoxin-NADP leaf isozyme 1 and 2 chloroplastic (A0A0D3E2R1; A0A0D3DQI2) and mitochondrial outer membrane protein 4 (A0A0D3BZ29).

Several increased proteins in susceptible plants were involved with responses to abscisic acid (ABA), while in resistant plants these proteins showed decreased abundance. ABA is a phytohormone, known as a signaling molecule, responsible for the regulation of abiotic stress response (Taiz et al., 2017). Studies have shown that ABA can suppress the plant immune response, (Kim et al., 2011; Desclos-Theveniau et al., 2012) and in many pathosystems, this phytohormone can act antagonistically to the salicylic acid (SA) pathway. SA is another important phytohormone that can confer plant resistance against pathogens (Audenaert et al., 2002; Jiang et al., 2010). Furthermore, ABA can suppress the MAPK pathway, causing immunosuppression in A. thaliana and possibly in other cruciferous plants (Mine et al., 2017). ABA’s effect during plant-pathogen interaction is considered complex, however, is possible that the increased abundance of proteins involved with ABA response can be a mechanism, which can favor susceptibility (Kim et al., 2011; Desclos-Theveniau et al., 2012).

The increase of ABA can also lead to the accumulation of other proteins such as aquaporins. In this study, the aquaporins PIP3 (Q9FU0C0) and BoPIP1b1 (Q9FUL1) were differentially abundant; the second was evaluated by qRT-PCR and showed a downregulation trend in both cultivars. These aquaporins were identified in chloroplast samples with increased abundance only in the susceptible plants. PIP aquaporins are intramembrane channels important for the transport of water and CO_2 in the plant tissues (Luu and Maurel, 2005; Verkman, 2013).

The detection of these proteins in chloroplast samples was not expected, however, since we sampled intact and broken chloroplasts, it is possible that some non-chloroplast proteins were also isolated. Our results suggest that the accumulation of these proteins may be related to ABA, as observed by Aroca et al. (2006), in leaves of Phaseolus vulgaris after ABA treatment. Aquaporins are multifunctional and some isoforms are able to detect pathogen molecular patterns (PAMPs) such as harpins (Zhu et al., 2000; Flexas et al., 2007). The transport of hydrogen peroxide has also been associated to aquaporins (Taiz et al., 2017). It was demonstrated that the loss of function of the gene locus AtPIP1;4 in Arabidopsis cancels the import of apoplastic H_2O_2 induced by the pathogen and consequently blocks the plant immune response (Tian et al., 2016).

Curiously, another protein named epithiospecifier-like (ESP-like; A0A0D3CQ9U9) involved in defense response, was decreased in susceptible plants and unchanged in resistant plants. Conversely, the expression of ESP gene was upregulated according to qRT-PCR results in both cultivars (23-fold change in the resistant plant and 3-fold change in the susceptible). In the previous study performed by Ribeiro et al. (2018), another resistant cabbage cultivar (União) was analyzed and ESP protein was exclusively identified in the resistant cultivar infected with Xcc by 2-DE analysis, demonstrating that the regulation of ESP protein can be important for the plant defense against Xcc. The ESP protein is related to the glucosinolate pathway involved in plant protection against herbivory pests. Glucosinolates are secondary metabolites, known as phytoanticipins (preformed antimicrobial compounds), representing one of the first chemical barriers against pathogen attack (Osborn, 1996). These metabolites can be found extensively in Brassicaceae plants (i.e., broccoli, cabbage, mustard), and are biologically active compounds reported in some processes of plant defense including stress response and antioxidant activities (Bennett and Wallsgrove, 1994; Halkier and Gershenzon, 2006).

**Interaction Model of Resistant B. oleracea–Xcc Interaction**

Overall, in this study, we observed that the protein profiles of the resistant and susceptible plants were similar, especially regarding the predominant GO terms. However, a higher number of pathogen-related proteins were identified in the resistant plants and therefore we propose a model of this interaction based on protein localization and their role in the cell (Figure 3 and Table 1). This model can help better understand the plant response to Xcc infection and provide candidate genes for the development of more efficient pathogen control strategies.

The classical mechanism of bacterial recognition occurs at the beginning of infection, in an attempt to neutralize the effectors released by the bacterium and repair the damage caused in the cell. In the resistant cultivar Astra, we found several proteins with increased abundance probably involved in this initial response (see step 1 in the model), such as Lectin-like protein (A0A0D3CJFY3), leucine-rich repeat receptor kinase (A0A0D3D909), leucine-rich repeat receptor-like serine threonine-kinase (A0A0D3CSX5). These transmembrane signaling proteins, together with other proteins, such as NB-LRR,
FIGURE 3 | Schematic view of a model proposed with the proteins identified in the resistant *Brassica oleracea*–*Xanthomonas campestris pv. campestris* (Xcc) infected leaf and chloroplast-enriched proteomes. The figure shows the localization, as proposed by UniProt database; detailed information on the proteins is presented in Table 1. The names in black and blue indicate proteins with increased and decreased abundance, respectively. The steps begin at the recognition of the pathogen, involving important signaling proteins, activation of molecular defense response pathways and oxidative stress response (steps 1–3), followed by UPS (ubiquitin pathway system) modulation and repair proteins (step 4), as well as alteration of metabolic and photosynthetic pathways (steps 3 and 4).

can be essential to sense the pathogen and promote systemic immunity (O’Neill and Bowie, 2007; Couto and Zipfel, 2016). Other signaling proteins identified showing increased abundance were the universal stress protein A (A0A0D3CN30) and universal stress PHOS34-like (A0A0D3CTQ3). Although the exact role of PHOS34 in plant defense is not known, studies have reported that this protein can be phosphorylated by MPK3 and MPK6, and after treatment with the flagellin fg22 peptide (Merkouropoulos et al., 2008), suggesting that this protein may be related with cellular signaling in the presence of the bacterium.

Several antioxidant/detoxification proteins were also increased, including superoxide dismutase Fe (F8U7Z7), peroxidase (A0A0D3C7R8), peroxiredoxin (A0A0D3DSN3; A0A0D2ZZRQ6; A0A0D3BYD5) and glutathione S-transferase U5 and U19 (A0A0D3BT71; A0A0D3CVZ5). The accumulation of ROS can be toxic to the pathogen by inhibiting and/or reducing its survival (Jones and Dangl, 2006; Zhang and Zhou, 2010). However, ROS accumulation can lead to the oxidation of important cell components like lipids and genetic material (Sharma et al., 2016). An intriguing result obtained was the decrease of other extra and intracellular antioxidant proteins including glutathione peroxidase mitochondrial; (A0A0D3AT05; A0A0D3DQE3) peroxidase 3-like (A0A0D3C7R9); peroxiredoxin-mitochondrial (A0A0D3CMF0) and peroxidase 32 (A0A0D3E2V6) in the resistant interaction. This result may indicate that a balance in the abundance of proteins related to oxidative stress, maintaining some proteins with increased abundance and others with decreased abundance may be important for an efficient control of the pathogen without extensive damage to the plant tissue.

Once the pathogen overcomes the first line of defense, other events occur in response to the effector delivery into the cell by the type III secretion system. At this stage, proteins such as NB-LRR proteins interact with the pathogen effectors (Spallek et al., 2009; Marino et al., 2012). In this study, we identified a leucine-rich repeat receptor kinase PEPR1 (A0A0D3D099), which has been reported as the receptor for AtPep1, a peptide elicitor from *Arabidopsis* that signals activation of innate immune response against pathogens (Yamaguchi et al., 2006) as well as a probable LRR
receptor-like serine/threonine-protein kinase At1g29720 RFK1-like (A0A0D3CSX5). Both proteins are integral components of the membrane (model-step 1) and may be involved in triggering a defense response.

Ubiquitination pathway also seems to play an important role in the resistant interaction. The protein 14-3-3-GF14 kappa (A0A0D3BET0), known as a metabolism regulator associated with abiotic stress, was identified and can modulate other proteins by facilitating their degradation by ubiquitins (Fuller et al., 2006; Chang et al., 2009; Liu et al., 2017). The ubiquitin pathway is necessary to tag proteins that should be degraded, however, bacterial effectors may also interact with ubiquitin proteasome system (UPS) as a false system protein (Figure 3). The bacterial effectors can be ubiquitinated and degraded by proteasomes; they can also interfere in the system, act as a ubiquitin ligase or inhibit the specific UPS steps (Collins and Brown, 2010). Proteins related to ubiquitination showed reduced abundance in the present work (A0A0D3CJZ8; A0A0D3ARJ5, A0A0D3ECQ3, A0A0D3BLH4). In a highly resistant plant, ubiquitination proteins also showed reduced abundance at 24 hai (Ribeiro et al., 2018), which may indicate a negative regulation of this pathway, leading to cell death and consequently resulting in limitation of bacterial growth (Spallek et al., 2009; Marino et al., 2012).

Proteins involved with defense against pathogens were also increased including Ferredoxin–NADP leaf isozyme 1 and 2, chloroplastic (A0A0D3E2R1; A0A0D3DQI2) and annexin (I3Y171). Annexins are members of a well-known family of proteins involved in tolerance against environmental stresses and have been studied in tobacco, cotton, Brassica and Arabidopsis plants (Jami et al., 2008; Konopka-Postupska et al., 2009; Clark et al., 2012; Szalonek et al., 2015). AIG2 (A0A0D3BZV5) is another defense protein, which has not been functionally characterized yet, however, it is known that the corresponding gene is induced in Arabidopsis by the avirulent gene avrRpt2 of Pseudomonas syringae (Reuber and Ausubel, 1996).

Defense response is also highly correlated with the levels of phytohormones, such as JA, ethylene (ET), ABA, and cytokinin. JA is important in the plant defense against various stresses. As seen in the model, the indirect activation of JA by the octadecanoid pathway and H2O2 accumulation can result in the activation of biotic stress response. The JA pathway can activate other pathways such as the signal transduction pathway, inducing the formation of chemical and physical barriers against pathogen or herbivore attacks (Kazan and Manners, 2008). In addition to the lectin proteins identified, plastid lipid-associated protein 2 and 3, chloroplastic (A0A0D3A546; A0A0D3BRT9) were also identified.

Another phytohormone involved in defense signaling is cytokinin, involved in plant development, cellular differentiation and senescence (Hwang et al., 2012). It has been reported that high levels of this hormone increased plant immunity (Swartzberg et al., 2008; Choi et al., 2010; Argueso et al., 2012). In this study, some proteins responsive to cytokinins were increased including 50S Ribosomal Chloroplastic protein.
of the nucleotide sequences of both genes (endogenous and BoCHB4 like proteins that cleave chitin molecules. In general, studies have shown increased abundance in the catabolic process in the cell (Stintzi et al., 1993). Proteins, are members of the chitinase family that participate in the molecular analysis and high gene expression levels. Endochitinase-like proteins, the CHI-B4 like protein, as mentioned above, showed increased abundance in both cultivars. qRT-PCR analysis showed that the corresponding gene was upregulated sevenfold in the resistant cultivar and twofold in the susceptible one. Taken together, the model presented here can represent a step-by-step of the defense mechanism in resistant brassica plants, beginning at the recognition of the pathogen, with the activation of important signaling proteins, molecular defense response pathways, and oxidative stress response (steps 1–3 in the model), followed by UPS modulation and repair proteins (step 4), and alteration of metabolic and photosynthetic pathways (steps 3 and 4). The model may also contribute to better understand the molecular responses during the plant–pathogen interaction reflected by the differential abundance of proteins under Xcc infection.

Functional Validation by Overexpression of CHI-B4 Like Protein in A. thaliana

In this study, several candidate proteins were identified, potentially involved in the resistance response to Xcc. One of these proteins, the CHI-B4 like protein, as mentioned above, showed increased abundance in the proteomic analysis and high gene expression levels. Endochitinase-like proteins, are members of the chitinase family that participate in the catabolic process in the cell (Stintzi et al., 1993). The chitinases are also classified as pathogen related proteins (PRs) and endochitinases belong to group 3 of PRs that cleave chitin molecules. In general, studies have related chitinases to plant–fungus interaction (reviewed by Jääl et al., 2015).

A. thaliana plants overexpressing cabbage BoCHB4 gene under the control of CaMV 35S promoter were obtained through transformation. The presence of the transgene was confirmed in five homozygote plants and single copy insertions were observed by Southern blot analysis for each positive event (Figure 4). The transcript level of the transgene was also assessed, and the transgenic lines showed a relative expression level 588-fold higher than the wild type (Col-0). Since CHI-B4 like can also be found in A. thaliana, an alignment of the nucleotide sequences of both genes (endogenous and transgenic) was performed and showed 84% identity with the Arabidopsis gene, and therefore the expression levels detected were probably related to the transgene and not to the endogenous gene.

A phenotypic evaluation of A. thaliana plants overexpressing BoCHB4 was performed and at 48 hai the WT plants began to show the first symptoms (Figure 4). The phenotypic analysis of the WT and transgenic lines after inoculation of Xcc showed that at 5 days after inoculation (dai), among the surviving plants, the WT replicates were almost totally necrotic and most leaves were dead, while the transgenic plants showed no symptoms even at 15 days after inoculation. According to the disease scoring index based on Meyer et al. (2005), A. thaliana WT Col-0 was highly susceptible, showing severe necrosis and leaf death, while the transformed plants were highly resistant, since no symptoms were observed. Based on these results, we conclude that the overexpression of BoCHB4 can confer resistance against the bacterial pathogen Xcc. This work provides an important contribution regarding the comprehension of resistance mechanisms and offers candidate genes to be used in genetic breeding programs aiming at the development of more efficient strategies for black rot disease control.

AUTHOR CONTRIBUTIONS

CS performed and designed the experiments, analyzed and interpreted data, and wrote the manuscript. FN, GD, WF, and JJ-N assisted in proteomic data acquisition and analysis. GP, PH, VS, OO-N, and MG-d-S assisted in gene isolation, vector design, and plant transformation. OF analyzed the data and revised the manuscript and AM designed the experiments, wrote the manuscript, and lead the work.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2019.00414/full#supplementary-material

**FIGURE S1** | Workflow showing the entire procedure for sample preparation.

**FIGURE S2** | SDS-PAGE step prior to LC-MS/MS analysis. The section indicated by the red square was excised and submitted to in gel digestion using trypsin.
proteomic analysis.

(a) Quantitative distribution of proteins from inoculated leaves (LPI) and from the control condition (LPC) as well as from chloroplast-enriched inoculated samples (ChlRI) and from the control condition (ChlRC) in the resistant interaction.

(b) Quantitative distribution of proteins from the susceptible inoculated leaves (LS) and from the control condition (LSC) as well as from chloroplast-enriched inoculated samples (ChlSI) and from the control condition (ChlSC) in the susceptible interaction.

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