GENE EXPRESSION RESPONSE OF *ARABIDOPSIS THALIANA* TO INOCULATION WITH *PECTOBACTERIUM CAROTOVORUM* subsp. *CAROTOVORUM*

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SUMMARY

Pectobacterium carotovorum subsp. *carotovorum* (*Pcc*) is a soft rot bacterial pathogen of a wide range of plant hosts. Under pathogen attack, plants respond locally as well as systemically by activating a broad spectrum of defense mechanisms. Systemic acquired resistance (SAR) as a response following inoculation of plants with *Pcc* is still poorly understood. Using the model plant *Arabidopsis thaliana*, we investigated the molecular basis of SAR following inoculation with *Pcc*. Five-week-old *A. thaliana* plants were inoculated with a lysogeny broth culture of *Pcc*, and leaves were sampled 24, 48 and 72 h after inoculation. The expression profiles of the plant defense genes: serine/threonine kinases, F-box, protease inhibitor, pathogenesis-related protein 1, phenylalanine ammonia lyase, Myb transcription factor, superoxide dismutase 1 and lipoxygenase were determined by real-time PCR to unveil the SAR mechanism after 24, 48 and 72 h after inoculation. The results revealed that *Pcc* inoculation triggered a genetic response in *A. thaliana,* which resulted in the differential expression of these selected plant defense genes. This suggests that these genes play an important role in the mechanism of response to *Pcc*.

Keywords: expression profiles, *Pectobacterium carotovorum*, real-time PCR, systemic acquired resistance.

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INTRODUCTION

Pectobacterium carotovorum subsp. *carotovorum* (*Pcc* or *P.c.* subsp. *carotovorum*) formerly known as *Erwinia carotovora* subsp*. carotovora*, causes soft rot disease in a wide range of economically important crops. During infection, *Pcc* produces plant cell wall-degrading enzymes (PCWDEs), which include pectinases, cellulases and proteases, to macerate tissue upon contact (Pérombelon and Kelman, 1980). Furthermore, cellular contents ooze, and the pathogen proliferates as it exploits the nutrients released from the colonised tissue. Finally, this leads to the development of a soft rot symptom (Kotoujansky, 1987; Barras *et al*., 1994). During disease progression the plant responds by activating the signal transduction mechanism (Palva *et al*., 1993). In *Arabidopsis*, PCWDEs trigger a defense response, which is dependent on JA/ET signaling pathways, independent of salicylic acid (Norman-Setterblad *et al*., 2000). It was subsequently shown that *Pcc*-derived elicitors, HrpN and PehA, triggered defense responses and induced both SA- and JA/ET-dependent signal pathways in *Arabidopsis* (Kariola *et al*., 2003).

Plants respond to pathogen attacks by using a large array of defense responses, which include their basal resistance and induced mechanisms, both locally and systemically (Djami-Tchatchou *et al*., 2013). SAR confers long-lasting and effective enhanced disease resistance at the whole plant level to subsequent infection by pathogens including viruses, bacteria, oomycetes and fungi (Mishina and Zeier, 2007; Djami-Tchatchou *et al*., 2015). The induction of the plant defense by exoenzyme PCWDEs treatment confers increased resistance against subsequent infection by *Pcc* in the tobacco plant (Palva *et al*., 1993). It was also shown that the plant-derived compounds capsaicin and jaceosidin, derived from native Korean plant species, trigger induced resistance against *Pcc* in tobacco (Song *et al*., 2013). Furthermore, with the use of a priming agent it was reported that the chemical β-aminobutyric acid enhances *Arabidopsis* resistance against *Pcc* and the resistance is correlated with a pattern-triggered immunity (Po-Wen *et al*., 2013).

Plants have evolved their own powerful defense mechanisms including a sophisticated molecular system for pathogen perception, and the activation of structural

and biochemical defense mechanisms to protect them against pathogen attacks (Djami-Tchatchou *et al*., 2013). Following pathogen recognition, some genes like *F-box* proteins, a protein motif of approximately 50 amino acids that function as a site of protein-protein interaction, are involved in the signal transduction, which results in the induction of gene expression (Craig and Tyers, 1999; Van Loon *et al*., 2006). During signal transduction, the serine/threonine kinases play a regulatory function through the enzymatic protein phosphorylation at serine/threonine residues (Dangl and Jones, 2001). In tomatoes it was shown previously that resistance to the bacterium *Pseudomonas syringae* is mediated by the specific interaction between the plant serine/threonine kinase *Pto* and the bacterial protein Avr Pto (Sessa and Martin, 2000). The transcriptional activation of these defense genes represents a crucial part of the plants defence mechanism against pathogens following pathogen or elicitor perception (Djami-Tchatchou *et al*., 2013, 2015). Transcription factors (TFs) naturally act as master regulators of many crucial biological processes such as: responses to biotic and abiotic stresses, development, differentiation, metabolism, defense, etc. (Ambawat *et al*., 2013). For instance, the *Myb* TFs, which have been intensely studied in numerous plants and model plants such as *A. thaliana* represent a large family involved in the regulation of gene expression during plant biotic and abiotic stress responses and defense reactions (Lippold *et al*., 2009; Liu *et al*., 2008). The network association of the signal transduction pathways combined with the transcriptional activation contributes to the establishment of the SAR by inducing the defense response genes (Zeier, 2005).

In addition, several studies indicated that Pathogenesis Related proteins 1 (PR1) have routinely been used as a molecular marker of SAR, and they contribute to increase pathogen resistance by directly exerting harmful effects to microbial invaders during SAR (Návarová *et al*., 2012). Recently, a study demonstrated that PR1 played a crucial role in the disease resistance of *A. thaliana* to the necrotrophic bacterium *Pcc* following the priming action of the β-aminobutyric acid (Po-Wen *et al*., 2013). In potato tuber it was shown that the pectin enzymes of *Erwinia carotovora* subsp. *carotovora* increased the expression of a number of plant defense genes such as the phenylalanine ammonia lyase (*PAL*), one of the markers of plant defense responses, and contributed to the response to soft-rot disease (Yang *et al*., 1992). Another study revealed that the increase expression of *PAL* in rice resulted in the resistance to the rice bacterial blight *Xanthomonas oryzae* pv. *oryzae* strain T7133 (Ai-Hua *et al*., 2005). This study also showed that *lipoxygenase* (*LOX*), which catalyses the oxygenation of polyunsaturated fatty acids to form hydroperoxides, also played and contributed to the resistance to the rice bacterial blight. Another class of plant defense gene is protease inhibitors detectable in leaves in response to the attack of pathogenic microorganisms which are able to inhibit the growth of a variety of pathogenic bacterial and fungal strains (Ryan, 1990; Kim *et al*., 2009).

Several other approaches such as gene expression profiling have been used to study a plant's response to a pathogen, including serial analysis of gene expression (Gyetvai *et al*., 2012), cDNA-AFLP (Colling *et al*., 2013), microarrays (Allie and Rey, 2013), RNAseq (Djami-Tchatchou *et al*., 2012) and real-time PCR (qPCR) quantification techniques (Djami-Tchatchou *et al*., 2015). Quantitative real-time PCR is considered the standard technique for the accurate, sensitive and rapid measurement of gene expression (Derveaux *et al*., 2010). It accurately quantifies starting amounts of DNA or cDNA targets, and is based on the method of PCR with the resulting PCR products monitored in real time, whereas the amplification process is monitored by fluorescence technology (Valasek and Repa, 2005). To the best of our knowledge no study has been conducted to investigate the responses of *A. thaliana* to inoculation with *Pcc* without a priming agent. Furthermore, based on their important functions in plant response to pathogen attacks previously reported (Yang *et al*., 1992; Sessa and Martin, 2000; Ai-Hua *et al*., 2005; Van Loon *et al*., 2006; Kim *et al*., 2009; Návarová *et al*., 2012; Ambawat *et al*., 2013), we selected and focused on a unique set of genes, namely serine/threonine kinases (*Ser/Thr*), *F-box*, protease inhibitor, pathogenesis-related protein 1 (*PR1*), phenylalanine ammonia lyase (*PAL*), *Myb* transcription factor, superoxide dismutase 1 (*SOD*) and lipoxygenase (*LOX*) to study their expression in response to the infection of *A. thaliana* with *Pcc*.

MATERIALS AND METHODS

Bacterial strain. *Pectobacterium carotovorum* subsp. *carotovorum* strain BD163, used in this study, was obtained from the Plant Pathogenic and Plant Protecting Bacteria collection at the Agricultural Research Council, Plant Protection Research Institute, Pretoria, South Africa.

The strain BD 163 was isolated in 1999 from a potato plant showing the soft rot symptoms. At the time, the strain was characterised by using morphological and physiological features as well as biochemical tests. The bacterium was a Gram-negative rod, non-pigmented on nutrient agar, pectolytic on CVP medium, oxidase and indole negative, strongly fermentative and caused soft rot on potato slices. The results of these tests are characteristic for bacteria belonging to the genus *Pectobacterium* (Goszczynska *et al*., 2000). The isolate was identified as *Pcc* based on the utilisation of 95 carbon sources in Biolog GN2 micro-plates and comparison with the Microlog 4.2 databases (Biolog Inc., Hayward, CA). The similarity index was recorded at 0.96. The strain has not been characterised by molecular analyses.

BD 163 was grown in lysogeny broth and 12-hour-old culture with approximately 10^8 cells per millilitre was used to inoculate *A. thaliana*.

Table 1. Nucleotide sequence of primers designed for gene expression and amplification of bacterial BD 163 genes.

Genes	Forward Primer Sequence (5'-3')	Reverse Primer Sequence (5'-3')	Tm (ºC)
Serine/threonine kinases (AT1G69220)	AGACTCGGAAATTGGATCCTTCC	ATGCTATCCGGAAGAGACGTAG	60
Myb transcription factor (AT1G74840)	TCCGGTTATGCAAGTCTTTCCC	AGTGAGAGGTTGAGAGAAAGCG	60
F-box (AT4G28370)	GCCTGCGGTGAACTTATGCTC	CCCATTGCCCTGAATCAAGCC	60
Protease inhibitor (AT2G45180)	TGGTGTTTGCGCAGACTTATTG	GACATTGGCTTTAAGAGCGGTG	60
Pathogenesis-related protein 1 (AT2G14610)	ATGAATTTTACTGGCTAT	AACCCACATGTTCACGGCGGA	60
Superoxide dismutase (AT1G08830)	GCGGTAGTGTTCATGGATTTA	ACAGCTATAAACCTGGCAATC	60
Phenylalanine ammonia lyase (AT2G37040)	ATCCTCTCAACTGGGGGAGCTG	CGCAGCCACTTGTCCAATGGT	60
Lipoxygenase (AT3G45140)	GTTGTTCCTCAAGGGGGTAGAG	TCGGAGAAGAATATCGGCTTGG	60
Actin 8 (Djami and Dubery, 2015)	CCCAAAAGCCAACAGAGAGA	CATCACCAGAGTCCAACACAAT	60
Elf 1α (Djami and Dubery, 2015)	CACCACTGGAGGTTTTGAGG	TGGAGTATTTGGGGGTGGT	60
16S rRNA gene (Weisburg *et al*., 1991)	CCGAATTCGTCGACAACAGAGTTTGATCCTGGCTCAG	CCCGGGATCCAAGCTTAAGGAGGTGATCCAGCC	
GyrB genes (Brady *et al*., 2008)	TAARTTYGAYGAYAACTCCYTAYAAAGT	CMCCYTCCACCARGTAMAGTT	

Amplification and sequencing of the 16S rRNA and *gyrB* genes. Genomic DNA of BD 163 was extracted by using the GenElute Bacterial Genomic DNA Kit (Sigma, Germany), according to the manufacturer's instructions. The DNA was stored at –20ºC until further analysis.

The 16S rRNA gene fragment was amplified using the universal primers fD1 and rD1 (Table 1) as described by Weisburg *et al*. (1991). The *gyrB* genes were amplified using primers gyrB-01F and gyrB-02R (Table 1) as reported by Brady *et al*. (2008). PCR amplifications were performed in a DNA Thermal Cycler 480 (Perkin-Elmer Corp., USA). PCR products were purified with the ExoSAP PCR cleanup kit (Affymetrix, Santa Clara, CA, USA). Inqaba Biotechnology, Pretoria, South Africa, sequenced purified PCR products. The GenBank databases were used for homology searches using the BLASTn programme to check the similarities at the nucleotide level. The partial sequences of the 16S rRNA and *gyrB* genes were submitted to the GenBank database (https://www.ncbi.nlm.nih.gov/).

Plant material and treatment of *A. thaliana* leaf tissues. The *A. thaliana* plants used in this study were derived from ecotype Colombia (Col-0). Five-week-old *A. thaliana* plants grown in a controlled greenhouse environment under a 10/14 h light-dark photoperiod were used for the experiment. *A. thaliana* leaves were pressure infiltrated with a blunt-ended syringe containing 10^8 CFU/ml of *Pcc* strain BD 163. Control plants were also syringe infiltrated with an equal amount of lysogeny broth. After inoculation, the plants were kept at 100% relative humidity and the leaves were harvested after 0 h (leaf control, C0), 24, 48 and 72 h post inoculation respectively, and kept at −80°C until further experimentation.

Total RNA extraction and cDNA synthesis. *A. thaliana* leaves were ground in liquid nitrogen and total RNA was extracted from 100 mg ground leaf tissue using the Trizol-reagent method (Invitrogen, Carlsbad, CA, USA). The extracted RNA was subjected to DNase treatment using DNase I (Thermo Scientific, Waltham, MA, USA). Concentrations were determined using a NanoDrop® ND-1000™ Spectrophotometer (NanoDrop Inc., Wilmington, DE, USA). The RNA integrity of all samples was examined by electrophoresis on a 1.5% agarose gel in a 1× Tris-Borate-EDTA (TBE) buffer containing 0.5 µg/ml ethidium bromide. The gels were visualised under UV light using a Bio-Rad Image Analyzer and Quantity One™ Version 4.6.1 Software (Bio-Rad Laboratories, Johannesburg, South Africa). Total RNA samples were aliquoted and stored at −80°C for later use. The DNase-treated RNA, isolated from leaves harvested at the different time points, were reverse transcribed to cDNA using a RevertAid™ Premium First Strand cDNA synthesis kit (Fermentas, Thermo Scientific, Waltham, MA, USA).

Gene expression analysis. Real-time PCR (qPCR) was used for the gene expression analysis. Selected genes included serine/threonine kinases (AT1G69220), *F-box* (AT4G28370), protease inhibitor (AT2G45180), pathogenesis related protein 1 (AT2G14610), phenylalanine ammonia lyase (AT2G37040), *Myb* transcription factors (AT1G74840), superoxide dismutase 1 (AT1G08830) and lipoxygenase (AT3G45140). These genes were selected

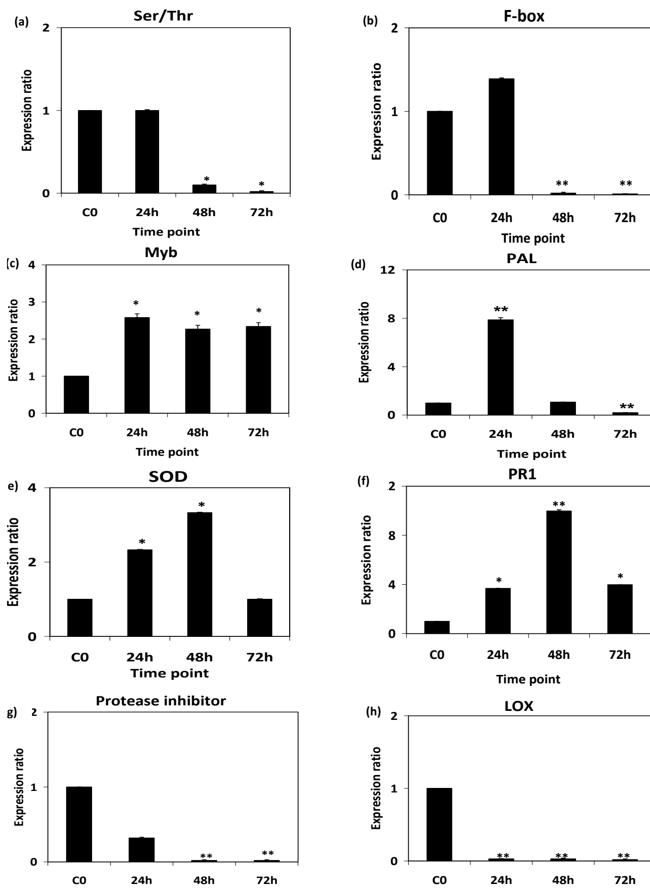

Fig. 1. Relative gene expression analysis of *A. thaliana* leaf tissue response to *Pcc* inoculation. The data were normalised using elongation factor 1-alpha and actin 8 to give the relative gene expression wherein error bars represent the standard error of mean. Expression analysis was performed on three biological repeats with three technical replicates of each. () Indicates no significant differences, with $P>0.05$, (*) indicates that there was a significant difference with $P<0.05$, and (**) indicates that there was a highly significant difference with $P<0.01$. Serine/threonine kinases (*Ser/Thr*), F-box, protease inhibitor, pathogenesis-related protein 1 (*PR1*), phenylalanine ammonia lyase (*PAL*), Myb transcription factor, superoxide dismutase 1 (*SOD*) and lipoxygenase (*LOX*).

based on their involvement in plant defense response mechanisms (Yang *et al*., 1992; Sessa and Martin, 2000; Alscher *et al*., 2002; Blee, 2002; Ai-Hua *et al*., 2005; Van Loon *et al*., 2006; Kim *et al*., 2009; Návarová *et al*., 2012; Ambawat *et al*., 2013). The resulting first strand of cDNA was diluted (1:10) and used for the qPCR experiment. The primer pairs of the selected target genes were designed using the Primer Quest tool (Integrated DNA Technologies, Coralville, IA, USA) from sequences obtained in the NCBI database (Table 1). qPCR was performed to analyse the expression of each gene on the Rotor gene-3000A instrument (Qiagen, Venlo, the Netherlands) using the FastStart essential DNA Green Master Kit (Roche, Mannheim, Germany) according to the manufacturer's instructions. The cycling conditions were as follows: initial denaturation for 10 min at 95°C, followed by amplification and quantification cycles repeated 40 times, each consisting of 5 s denaturing at 95°C, 10 s annealing at primer-specific temperatures and 20 s extension at 72°C. Three biological replicates were used with three technical replicates of each. The quantification of the relative changes in gene expression was performed, as described above, with elongation factor 1-alpha and actin 8 as reference genes with their primer sequences obtained from Djami-Tchatchou and Dubery (2015). qPCR data were statistically compared between untreated and treated samples at each time point on the statistical analysis software GraphPad inStat 3 (GraphPad Software, San Diego, CA, USA) using one-way ANOVA (Kuznetsova *et al*., 2010) with the confidence level of all analyses set at 95%, and values with $p<0.05$ considered to be significant.

RESULTS

Bacterial strain BD 163 identification: **amplification and sequencing of the 16S rRNA and *gyrB* genes.** The genomic DNA of BD 163 extracts were of high quality without degradation. The PCR products of 16S rRNA gene fragment and the gyrase B (*gyrB*) genes were successfully amplified and sequenced.

The obtained partial sequences of 16S rRNA (1374 bp) and *gyrB* (738 bp) genes were compared to other sequences present in Genbank using the BLASTN program to determine the similarity at the nucleotide level with other bacterial species. The selection criteria were based on: the high percentage of maximum identity (range of the percentage = 95-100%), the coverage of 100% and an E-value less or equal to 0. The BLAST analysis showed high similarities between our genes and those of *Pcc* strains present in the database. Some examples of the BLAST hits showing the similarities are confined in Table 2. The partial sequences of the 16S rRNA and *gyrB* genes were submitted to the GenBank database and the following accession numbers were allocated: KY610284 to the 16S rRNA sequence of BD 163 and KY612209 to the *gyrB* sequence.

Total RNA extraction and cDNA synthesis. Total RNA was extracted from 100 mg of leaf tissues obtained from the mock sample (C0) and the samples obtained after 24, 48 and 72 h post infection followed by DNase I treatment. The high-quality DNase-treated RNA obtained was confirmed firstly by the A_{260}/A_{280} absorbance ratio whose values were always between 2.12 and 2.15, indicating that RNA was relatively free of protein contamination; and the A_{260}/A_{230} ratio was higher than 2.0, indicating that RNA was of high purity and without polyphenol and polysaccharide contamination (Djami-Tchatchou and Straker, 2012). Furthermore, the RNA integrity was assessed by the sharpness of distinct 18S and 28S ribosomal RNA bands visualised on gel electrophoresis suggesting that RNA was not degraded and was also relatively free of RNases. Then

Table 2. Relative gene expression analysis of *A. thaliana* leaf tissue response to *Pcc* inoculation. The data were normalised using elongation factor 1-alpha and actin 8 to give the relative gene expression.

	Ser/Thr	*Myb*	*F-box*	*Protease inhibitor*	*PR1*	*SOD*	*PAL*	*LOX*
C0	1	1	1	1	1	1	1	1
24h	1 ± 0.01	2.58 ± 0.1	1.39 ± 0.01	0.32 ± 0.01	3.68 ± 0.01	2.33 ± 0.01	7.87 ± 0.18	0.03 ± 0.03
48h	0.1 ± 0.01	2.27 ± 0.1	0 ± 0.01	0.02 ± 0.01	9.98 ± 0.1	3 ± 0.01	1.07 ± 0.01	0.03 ± 0.01
72h	0.02 ± 0.01	2.34 ± 0.1	0.01 ± 0.03	0.02 ± 0.01	3.98 ± 0.01	1 ± 0.01	0.2 ± 0.01	0.02 ± 0.01

Values are expressed as mean ± S.E.M of three biological repeats with three technical replicates of each (one-way ANOVA)

the high-quality RNA obtained was reverse transcribed to cDNA.

Gene expression analysis. In order to profile the gene expression of *A. thaliana* in response to *Pcc* infection, after 24, 48 and 72 h post infection the quantitative expression analysis of eight genes, which included *Ser/Thr*, *F-box*, protease inhibitor, *PR1*, *PAL*, *Myb* transcription factor, *SOD* and *LOX*, was performed and normalised against elongation factor 1-alpha (*Elf 1α*) and actin 8 to give the relative gene expression wherein error bars represent the standard error of mean (Fig. 1 a-h; Table 2). After the normalisation steps the expression profiles of the control samples (CO) were set as calibrator and used to determine the expression fold of the treated samples with *Pcc* by dividing all the expression profiles by the expression profile of the control samples. Therefore, the expression fold of the CO became 1 and any expression fold of the treated samples higher than 1 was considered up regulated; any expression fold less than 1 was considered down regulated and any expression fold = 1 was considered not differentially expressed.

The qPCR quantification revealed that the transcript level of all the selected genes of *Arabidopsis* exhibited different expression kinetics at 24, 48 and 72 h, respectively, following inoculation with *Pcc* (Fig. 1 a-h).

At 24 h following inoculation with the bacterium, the qPCR results showed that the expression profile of protease inhibitor was not significantly down regulated and the expression profile of lipoxygenase was significantly down regulated with a minimum expression of about 30 fold compared to the control (Fig. 1 g, h). Still, at 24 h the expression profile of some genes such as *Myb*, *PAL*, *PR1* and *SOD* showed a significant up regulation and a non-significant up regulation for *F-box* (Fig. 1 b, c, d, e, f). The expression profil of *Myb* and *SOD* increased with the fold expression higher than 2; whereas the expression of *PR1* increased with a fold expression change higher than 3.5 compared to the control and the highest expression profile at 24 h was observed for *PAL* with a maximum expression higher than 7 compared to the control.

At 48 h, the qPCR results showed that the expression profiles of *Ser/Thr*, *F-box*, the protease inhibitor and *LOX* were significantly down regulated compared to the control plants (Fig. 1 a, b, g, h). The expression profiles of *Ser/Thr*, *F-box*, the protease inhibitor and *LOX* decreased significantly with the fold expression varying from 10-50 fold relatively low compared to the basal levels of the control. Whereas the expression profiles of *Myb* were significantly up regulated with 2 fold change, *SOD* with 3 fold change and *PR1* with a maximum fold change of 10 compared to the expression of the control sample (Fig. 1 c, e, f).

At 72 h, the expression profiles of *Myb* and *PR1* were significantly upregulated with 2 fold and almost 4 fold change respectively following *Pcc* inoculation, while the expression profiles of all the other genes except *SOD* (not differentially expressed) were significantly down regulated with the fold expression varying from 5-100 fold relatively low compared to the control (Fig. 1 a, b, d, g, h).

Thus, the qPCR results revealed that almost all the selected genes were differentially expressed following inoculation of *A. thaliana* with *Pcc*.

DISCUSSION

Pectobacterium carotovorum subsp. *carotovorum* is a phytopathogen, which causes economic losses in agriculture due to its wide host range. In this study the sequencing and BLAST analysis of 16S rRNA and *gyrB* genes of our bacterial strain BD163 confirmed that the strain BD 163 belongs to the species *Pectobacterium carotovorum* subsp. *carotovorum*.

Because of its genetic system, *Arabidopsis* has been a common plant of choice for the study of the genes involved in plant-pathogen interactions. Significant advances in functional genomic studies of recent years have improved our understanding of plant-pathogen interactions through studies pertaining to the expression profiles of defense genes leading to the hypersensitive response and SAR. Within this context the present study was undertaken to determine the expression profile of certain defense-related genes in *A. thaliana* leaves in response to inoculation with *Pcc*. Unlike mammals, plants do not have an adaptive immune system and therefore, they rely on innate immunity and systemic signals coming from the sites of infection (Sanabria *et al*., 2008). The pathogen can initiate a strong and rapid reaction in the host plant, which results in the induction of resistance genes to protect the plant against parasitic attacks (Djami-Tchatchou *et al*., 2013; Moeen and Akram, 2014). This study found that, in general, all

the selected genes *Ser/Thr*, *F-box*, protease inhibitor, *PR1*, *PAL*, *Myb*, *SOD* and *LOX* were differentially expressed in *A. thaliana* following inoculation with *Pcc*.

Plant perception of parasitic attacks is associated with networks of signal transduction pathways combined with transcriptional activation. In this study, no differential expression of *Ser/Thr* was observed at 24 h following *Pcc* inoculation (Fig. 1a), but a significant down regulation was observed in their expression at 48 and 72 h, respectively. These proteins have a key function in the regulation of signal transduction pathways through enzymatic protein phosphorylation at serine/threonine residues (Dangl and Jones, 2001). After pathogen recognition the activation of the signal transduction network results in a reprogramming of cellular metabolism, which leads to a large change in gene activity (Hammond-Kosack and Parker, 2003). The low expression pattern of *Ser/Thr* observed indicates that they were not involved in the signal transduction following *A. thaliana*'s perception of the *Pcc* bacterium but further investigation is needed for a clear conclusion.

The expression patterns of *F-box* showed a small non-significant expression increase at 24 h and a significant down regulation at 48 and 72 h, respectively, following inoculation with the bacterium (Fig. 1b). In plants, F-box proteins mediate hormone signalling, and are also associated with signal transduction (Craig and Tyers, 1999) and gene networks broadly regulated by microRNA-mediated gene silencing (Jones-Rhoades *et al*., 2006; Djami-Tchatchou and Dubery, 2015). A more recent study showed that the *Arabidopsis F-box-Nictaba* gene is a stress-inducible gene responsive to salicylic acid, bacterial infection and heat stress, and is involved in plant defense responses mediated by salicylic acid (Stefanowicz *et al*., 2016).

In addition, F-box proteins are also involved in various biological processes such as poly-ubiquitination, transcription elongation, centromere binding and translation repression (Zheng *et al*., 2002). The expression of *F-box* is important for plant pathogen response, as it is known that the activation of the signal transduction pathways results in the induction of gene expression (Van Loon *et al*., 2006). It was shown that the F-box protein is involved in the protein ubiquitination and degradation by the proteasome. The proteins that negatively regulate plant defense are targeted and degraded for the activation of defense responses during pathogen plant attack (Unver *et al*., 2013). In this regard, the significant down regulation of the F-box protein, observed at 48 and 72 h, respectively, suggests that they were either not involved in the ubiquitination process or there was no negative regulator of defense during the response of *A. thaliana* to *Pcc* infection.

The expression profile showed that the *Myb* transcription factor known to be involved in transcriptional activation was significantly upregulated following the inoculation of *A. thaliana* with *Pcc* at all time points (Fig. 1c). It is well demonstrated that transcription factors play important roles in the activation and fine-tuning of plant defense in response to pathogen attack, by either regulating specific genes or a cluster of genes (New *et al*., 2015). Dubos *et al*. (2010) report that in the *Arabidopsis* plant, Myb proteins are key factors involved in the regulatory networks controlling plant development, metabolism and responses to biotic and abiotic stresses. Based on previous findings and from the qPCR results, the pattern of expression of Myb proteins observed indicates that they are involved in transcriptional activation to trigger the defense response of *A. thaliana* to inoculation with *Pcc*. Similarly, in a previous study the up regulation of the *Myb* transcription factor enhanced the resistance of *Arabidopsis* to the bacterium *Pseudomonas syringae* infection (Seo and Park, 2010). Another study showed that *Myb72* induction played a crucial role in *Arabidopsis*-induced systemic resistance triggered by the rhizobacterial strains *Pseudomonas fluorescens* and *Trichoderma asperellum* T34 (Segarra *et al*., 2009).

PAL was highly expressed at 24 h, with a maximum expression higher than 7 fold change (Fig. 1d). *PAL* is a very important enzyme that links the primary and secondary metabolisms and it is involved in the deamination of phenylalanine to trans-cinnamic acid the initial step in the biosynthesis of various phenylpropanoids, coumarins, flavonoids and lignins (Mizuno *et al*., 2012). *PAL* also plays a crucial role in the biosynthesis of certain classes of phytoalexins, which are antimicrobials used by plants to protect themselves against pathogen invasion (Lamb *et al*., 1989). Therefore, due to *PAL* function in lignin biosynthesis, and knowing that lignification is a mechanism for disease resistance in plants, which results in the reinforcement of the cell wall to prevent pathogen invasion (Bhuiyan *et al*., 2009), the results of this study indicate that *PAL* may play an important role in *A. thaliana*'s defense response to *Pcc* infection. This might occur by slowing the spread of the pathogen to other parts of the plant though lignification during early response, which explains its strong expression at 24 h. A previous study found that the expression of *PAL* was induced in rice, which resulted in the activation of its defense against bacterial blight (Sha *et al*., 2005).

The expression level of *SOD* was significantly up regulated at 24 and 48 h, respectively, following the inoculation of *A. thaliana* with *Pcc* (Fig. 1e). In higher plants, *SOD* constitutes the first line of defense against reactive oxygen species (ROS) by acting as antioxidant and protecting cellular components from being oxidised by ROS (Alscher *et al*., 2002). Abiotic and biotic stress such as pathogen attack can induce the production of ROS, which can denature enzymes, oxidise lipids and fragment the DNA of the host plant (Raychaudhuri and Deng, 2008). We hypothesise that the invasion of *A. thaliana* by *Pcc* could have resulted in an enhanced production of ROS. Based on the crucial detoxification function of *SOD*, our results suggest that the *SOD* was highly expressed at 24 and 48 h, respectively, which could be to limit the formation of ROS and their removal from the plant cell.

The activation of signal transduction pathways enhances the expression of genes related to disease resistance, including pathogenesis-related protein-encoding genes. In this study an important category of genes, which was highly expressed is *PR1*, which showed significant upregulation at all time points with a maximum expression of 10 fold change at 48 h (Fig. 1f). PR1 proteins are a useful molecular marker for SAR and the salicylic-acid (SA) signaling pathway, and during SAR they contribute to enhance pathogen resistance by inflicting harmful effects directly onto microbial invaders or by attacking molecules in the cell wall of a bacterium or fungus (Návarová *et al*., 2012). PR1 proteins are produced following the perception of pathogen-derived molecules by host plant cells and the triggering of transduction pathways (Van Loon and Van Strien, 1999). Therefore, our result indicates that PR1 proteins were expressed after *Pcc* inoculation downstream of the initial signal transduction cascades, which involve SA signaling. A similar pattern was observed in a previous study, where it was shown that the upregulation of *PR1* led to the resistance of *Arabidopsis* to the bacterium *Erwinia chrysanthemi* (Fagard *et al*., 2007). Another study showed that priming *Arabidopsis* with the chemical β-aminobutyric acid resulted in the high expression of *PR1*, which enhanced the resistance of *Arabidopsis* to *Pcc* infection. This confirms that *PR1* is involved in the defense response mechanism of *A. thaliana* to *Pcc* infection (Po-Wen *et al*., 2013).

Lastly, we found that the expression patterns of the protease inhibitor and *LOX* were significantly repressed during *A. thaliana*'s response to *Pcc* inoculation (Fig. 1 g, h). *LOX* genes in plants are involved in a number of biological processes such as growth and development, pathogen resistance, senescence and responses to wounding (Porta and Rocha-Sosa, 2002). *LOX* is involved in the biosynthesis of oxylipin, a potent signaling molecule found in the defense reactions of plants (Blee, 2002). *LOX* can also catalyse the oxygenation of polyunsaturated fatty acids to produce fatty acid hydroperoxides, which may form free radicals that can promote cell death (Turner *et al*., 2002). The study by Sha *et al.* (2005) reports that the expression of *LOX* was induced and lasted up to 12 h following exposure to *Xanthomonas oryzae*, and conferred resistance to rice bacterial blight. However, it was also reported that hydroperoxides (the primary products of *LOX*) are highly reactive and may cause oxidative membrane damage leading to cell necrosis and premature cell death (Hildebrand, 1989). Since we found that the high expression of *SOD* observed in this study was to limit the formation of ROS produced during *Pcc* infection and their removal from the plant cell, we hypothesise that the downregulation of *LOX* observed was to limit the formation of ROS in order to avoid host cellular damage and enhance the response of *A. thaliana* to *Pcc* infection. Similarly, during apple flower interaction with the bacterium *Erwinia amylovora*, the expression of *LOX* was highly downregulated and resulted in the resistance of apple flower to the bacterium (Sarowar *et al*., 2011).

It was also reported that the growth of a variety of pathogenic bacterial and fungal strains can potentially be inhibited by protease inhibitors, also considered as antimicrobial peptides, from plants (Kim *et al*., 2009). In our study, however, we found that following the perception of *Pcc*, the expression of the protease inhibitor was not induced to contribute to the response of *A. thaliana* to bacterium inoculation.

This study aims to investigate the expression profiles of some selected defense-related genes of *A. thaliana* in response to *Pcc* infection. Our study showed that the differential expression of the selected genes plays an important role in the response of *A. thaliana* to *Pcc* infection, which can lead to disease resistance. The findings of our current study provide important new data in understanding the strategy used by *A. thaliana* to respond to *Pcc* infection. This understanding could contribute to develop molecular markers for soft rot resistance genes that may be used in plant breeding to produce more soft rot-resistant crop cultivars.

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