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Transcriptome profiling helps to identify potential and true molecular switches of stealth to brute force behavior in *Pectobacterium atrosepticum* during systemic colonization of tobacco plants

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Abstract In the present study, we have monitored the process of systemic plant colonization by the plant pathogenic bacterium *Pectobacterium atrosepticum* (*Pba*) using RNA-Seq analysis in order to compare bacterial traits under *in planta* and in vitro conditions and to reveal potential players that participate in switching from stealth to brute force strategy of the pathogen. Two stages of tobacco plant colonization have been assayed: i) the initial one associated with visually symptomless spread of bacteria throughout the host body via primary xylem vessels where bacterial emboli were formed (stealth strategy), and ii) the advanced stage coupled with an extensive colonization of core parenchyma and manifestation of soft rot symptoms (brute force strategy). Plant-inducible genes in *Pba* and potential players switching the pathogen's behavior were revealed. Genes from the *cfa* locus responsible for the production of coronafacic acid displayed the strongest induction in the asymptomatic zone relative to the symptomatic one and were shown experimentally to act as the true strategy "switchers" of *Pba* behavior *in planta*. Surprisingly, *cfa* genes appeared to be unnecessary for establishment of the asymptomatic stage of plant colonization but were required for the transition to soft-rot-associated symptomatic stage coupled with over-induction of jasmonate-mediated pathway in the plant.

Keywords Plant-microbe interactions · *Pectobacterium* · Soft rots · Virulence factors · Transcriptome profiling · Coronafacic acid · Bacterial emboli

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Introduction

Diseases caused by plant pathogenic bacteria result in large crop losses all over the world. Members of SRE (soft-rot *Enterobacteriaceae*) group, that includes *Pectobacterium* and *Dickeya* genus, are among the most devastating plant pathogens known to date (Charkowski et al. 2012; Mansfield et al. 2012). They deploy multiple plant cell wall (PCW) degrading enzymes (PCWDEs) that destroy plant tissues and are considered to be the major tool of brute force of SRE for successful plant host colonization (Perombelon 2002; Charkowski et al.

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2012; Tarasova et al. 2013; Davidsson et al. 2013; Hugouvieux-Cotte-Pattat et al. 2014). In addition, several virulence factors typical of biotrophic pathogens were revealed in *Pectobacterium* sp. allowing to suggest that these pathogens may also employ a stealth strategy for host colonization along with the brute force (Toth and Birch 2005; Toth et al. 2006; Liu et al. 2008).

Although considerable efforts have been applied to research virulence factors of SRE as well as their regulation (for review see Toth and Birch 2005; Charkowski et al. 2012; Davidsson et al. 2013), still only limited information is available on the quantitative and especially qualitative parameters of bacterial population *in planta*. Major emphasis of the research focused on plant-SRE pathosystems is given to invasiveness of bacteria and their ability to cause disease symptoms. As the result, population dynamics and processes of systemic plant colonization are poorly understood.

SRE are considered to predominantly colonize core parenchyma of the host, where they cause soft rot symptoms. However, several studies showed that members of both *Pectobacterium* and *Dickeya* genus extensively colonize xylem vessels (Czajkowski et al. 2010; Kubheka et al. 2013; Gorshkov et al. 2014; Moleleki et al. 2017). This allows bacterial cells to spread quickly both up and down the plant vessels, leading to systemic infection throughout the host organism. Interestingly, the number of *Pectobacterium* cells was shown to be higher below the inoculation site in comparison to tissues above it (Moleleki et al. 2017), implying the directed movement of bacteria to the underground part of the plant. This seems to be necessary for *Pectobacterium* to complete the life cycle within the host organism.

We have shown previously that downward migration of *P. atrosepticum* (*Pba*) during systemic plant colonization is, at least partially, caused by blockage of upward transpiration stream due to the formation of biofilm-like structures, which we proposed to call bacterial emboli (Gorshkov et al. 2014). Main (but not the only one) difference between a bacterial embolus and a typical biofilm is the requirement of plant polysaccharide (namely rhamnogalacturonan I, RG-I) for embolus formation (Gorshkov et al. 2016). During susceptible plant response, this polymer is released from PCW into the vessel lumen forming the initial extracellular matrix utilized by invading bacteria, which then is being substituted for the pectobacterial exopolysaccharide-containing one (Gorshkov et al. 2017a).

Bacterial emboli assemble well below the stem rot area and, therefore, extensive colonization of the vessels outstrips the settlement of bacteria in core parenchyma (Gorshkov et al. 2014). This suggests at least two alternative or sequential strategies employed by *Pba* during systemic plant colonization. The first strategy seems to be a stealth behavior when visually asymptomatic colonization of the xylem vessels by downward migration and formation of bacterial emboli occurs. The second, brute force strategy is employed when extensive colonization of core parenchyma and formation of soft rot occurs. It is reasonable to speculate that during stealth asymptomatic infections described frequently (Lapwood and Harris 1982; Helias et al. 2000; Perombelon 2002), pectobacteria are confined within the frameworks of the first strategy and do not exploit the second one. Therefore, dissecting the common and distinctive mechanisms of both stealth and brute force strategies is of a great importance for understanding *Pectbacterium* pathogenesis and for soft rot disease management optimization.

In this study, we tackle the aforementioned problem, by utilizing RNA-Seq analysis to compare transcriptomic profiles of *Pba* at two stages of systemic tobacco plant colonization with each other and also with *Pba* cultured in vitro. Tobacco plants have been chosen as host plants since the processes taking place in infected plant tissues during plant-*Pba* pathosystem formation were described thoroughly for tobacco plants (Gorshkov et al. 2014, 2016, 2017a), and this information is helpful for interpretation of RNA-Seq data. This approach led us to identify plant-inducible genes in *Pba* and potential players that participate in switching from stealth to brute force strategy of the pathogen during systemic plant colonization. Based on our RNA-seq data we were able to experimentally confirm the role of coronafacic acid as the true strategy "switcher" of *Pba* behavior *in planta*.

Materials and methods

Bacteria and plants growth conditions, plant inoculation, sample collection

A strain of *Pectobacterium atrosepticum* SCRI1043 (*Pba*) (formerly *Erwinia carotovora* ssp. *atroseptica* SCRI1043) and its *cfa* derivative were grown overnight in Luria–Bertani (LB) broth on a rotary shaker

(180 rpm) at 28 °C. *Nicotiana tabacum* cv. Petit Havana SR1 plants were grown axenically in test tubes placed in a growth chamber with a 16-h light/8-h dark cycle photoperiod. Seeds were surface-sterilized using diluted bleach (0.8% of active chlorine) and 1% sodium dodecyl sulphate for 30 min, washed seven times with sterile distilled water, then transferred to Murashige and Skoog medium (MS) in Petri dishes. Ten day-old seedlings were transferred to individual flasks containing MS.

Six to seven weeks after planting, the tobacco plants were infected with either wild type *Pba* or its *cfa* mutant. For plant inoculation, bacteria were grown until the early stationary phase (~2×10^9 colony-forming units, CFU ml^{-1}), then washed with sterile 10 mM $MgSO_4$ and resuspended in the same solution up to a density of ~2×10^7 CFU ml^{-1}. Sterile 10 mM $MgSO_4$ or bacterial suspensions containing ~2×10^5 cells were placed as 10-µl drops into the bosoms of the leaves in the middle part of the stems using sterile pipette tips and slight scratches were made simultaneously.

Two days after plant inoculation, different plant stem sections denoted in Fig. 1 were harvested for RNA-Seq analysis (only for the wild type *Pba*-infected plants), determination of bacterial CFU titre *in planta*, microscopy and immunocytochemistry analyses, and plant leaves (fully expanded leaves below the inoculation point) were harvested for plant gene expression analysis (Fig. 1). To determine CFU titre of bacteria *in planta*, different stem sections (Fig. 1) were weighed separately and ground in mortars with five volumes (*w*/*v*) of 10 mM $MgSO_4$. The number of CFUs was determined by the plating of serial 10-fold dilutions of the obtained suspensions onto 1.5% LB agar. The plates were incubated at 28 °C for 2 days before the CFUs were counted. Bacterial densities were presented as the log CFU per gram of colonized tissue. ggplot2 package was used for the visualization of CFU titre.

RNA extraction and cDNA library preparation

To extract total RNA, *Pba* cells from the aliquotes of suspensions used for plant inoculation were harvested (8000 g, 5 min, room temperature) and plant material (asymptomatic zone 1 and symptomatic zone 2 of the wild type *Pba*-infected tobacco plants, Fig. 1) was ground in liquid nitrogen in mortars. The obtained powder of infected plant sections or *Pba* cell pellets were resuspended in 1 mL of ExtractRNA Reagent (Evrogen, Russia) and the subsequent procedures were performed according to manufacturer's instructions. Residual DNA was eliminated by treatment of RNA samples with DNAse I using the DNA-free kit (Ambion). RNA quantity was analyzed using a Qubit fluorimeter (Invitrogen).

For RNA-Seq, total RNA (3 µg) was processed using Ribo-Zero rRNA Removal Kit (Plant) (Illumina) and then ScriptSeq™ Complete Kit (Bacteria)–Low Input (Illumina), which included bacterial rRNA depletion step, according to manufacturer's instructions. The quality and quantity of the cDNA libraries during processing before sequencing were monitored using the Agilent 2100 Bioanalyser (Agilent) and CFX96 Touch Real-Time PCR Detection System (Bio-Rad). Sequencing was conducted on an Illumina HiSeq 2500 platform. Each type of the libraries (asymptomatic zone 1 and symptomatic zone 2 of *Pba* infected plants as well as *Pba* cells grown in vitro) was sequenced in two biological replicates.

RNA-Seq data analysis

The quality of obtained reads was assessed using FastQC tool (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Bad quality bases of reads (Q-score < 30) as well as rRNA-corresponding and short (< 40 bp) reads were removed using Trimmomatic and SortMeRna software (Bolger et al. 2014; Kopylova et al. 2012). Coding sequences of *Pba* SCRI1043 genome were used as reference (ftp://ftp.ensemblgenomes.org/pub/bacteria/release-35/fasta/bacteria_68_collection/pectobacterium_atrosepticum/cds/Pectobacterium_atrosepticum.ASM69646v1.cds.all.fa.gz). The obtained read sequences corresponding to *Pba* genome can be accessed from NCBI's BioProject under the accession number PRJNA403794. Read pseudo-alignment and transcript quantification was performed using alignment-free kallisto tool (Bray et al. 2016). The analysis of the differentially expressed genes (DEGs) was carried out with edgeR package (Robinson et al. 2010). Genes with log2(fold-change) > 1 and significant differences in expression levels (FDR < 0.05) were considered as DEGs. DEGs were determined for all three possible comparisons: 1) asymptomatic zone 1 vs. symptomatic zone 2, 2) asymptomatic zone 1 vs. in vitro, 3) symptomatic zone 2 vs. in vitro.

Functional classification of *Pba* genes was performed according to CAZy (http://www.cazy.org/), DBD (http://www.transcriptionfactor.org/), UniProt (http://www.uniprot.org/), DOOR2 (http://csbl.bmb.

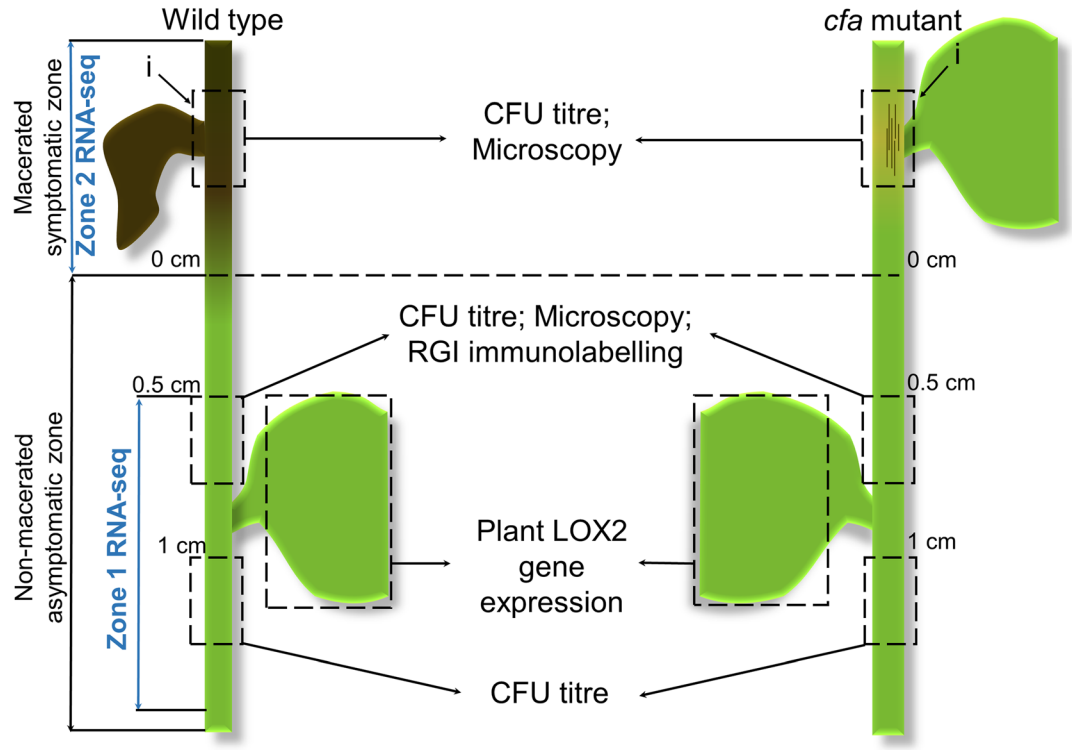


Fig. 1 Schematic representation of sampling locations for different analysis of tobacco plants systemic colonization by *P. atrosepticum* or its *cfa* mutant. In case of wild type bacterial infection, non-macerated asymptomatic stem area corresponds to initial stealth stage of systemic plant colonization, when bacteria colonize only xylem vessels and form bacterial emboli (Gorshkov et al. 2014). Macerated symptomatic stem area corresponds to advanced soft rot-associated stage when bacteria extensively colonize core parenchyma. Within these two areas, asymptomatic zone 1 and symptomatic zone 2 in plants infected by the wild type *Pba* were harvested for RNA-Seq analysis. (i) indicates the inoculation point. Corresponding stem zones of non-infected plants were taken as controls

uga.edu/DOOR/) and KEGG (http://www.genome.jp/kegg/) databases. To perform KEGG pathways/modules enrichment analysis, DEGs were separated into up- and down-regulated genes. KEGG pathways/modules significantly enriched by up- or down-regulated genes were determined by using Fisher exact test ($P < 0.05$). Both functional gene classification and enrichment analysis were carried out using modified custom R-scripts (http://github.com/RimGubaev/path_annotation, http://github.com/RimGubaev/deg_to_path). Classification of DEGs into operons was perfomed using DOOR2 database. The operons that contained 3 or more DEGs were visualized using ggplot2 package (http://ggplot2.org/) in RStudio; herewith, the following metrics were calculated: average, standard deviation (SD), median, interquartile range (IQR). The above procedures were carried out using custom R-script (http://github.com/RimGubaev/deg_to_operon).

Construction of the *cfa* mutant

To construct *cfa* mutant of *Pba*, *cfa6* gene sequence was PCR amplified from *P. atrosepticum* SCRI1043 with cfa6f and cfa6r primers (Table S15) and its internal *Nhe*I-*Sal*I fragment (1462 bp) was cloned into suicide vector pJP5603 (Penfold and Pemberton 1992). The resulting plasmid was mobilized into *Pba* from *Escherichia coli* BW 19851 (Metcalf et al. 1994) and *cfa* crossover clones were selected on kanamycin (20 μg/ml) containing plates. *cfa6* disruption was confirmed by PCR with combinations of primers to *cfa* and suicide vector sequences (cfa6f-cfa6r, cfa6f-pjp2 and cfa6r-pjp1, Table S15).

Gene expression analysis by qPCR

Total RNA from the leaves of control and infected with either the wild type *Pba* or its *cfa* mutant tobacco plants

(Fig. 1) was extracted, treated with DNAse and quantified as described above. 1 μg of RNA was used for cDNA synthesis using RevertAid reverse transcriptase (Thermo Scientific) according to manufacturer's instructions. Two μl of 5-fold-diluted cDNA were used as the template for qPCR.

qPCR was performed using the EVA-Green-containing master mix (Syntol, Russia) according to manufacturer's instructions. Primers for target and reference genes (Table S15) were designed using Vector-NTI Version 9 software (Invitrogen) and synthesized by Evrogen (Moscow, Russia). PCR was performed under the following conditions: 95 °C for 2 min, followed by 45 cycles at 94 °C for 10 s, 60 °C for 15 s and 72 °C for 30 s. After that, melt curve analysis was performed in a temperature range of 60–95 °C. The reactions were run and changes in fluorescence emission were detected using a CFX96 quantitative PCR system (Bio-Rad, USA). The amount of fluorescence was plotted as a function of the PCR cycle using CFX Manager Software (Bio-Rad, USA). The amplification efficiency (E) for all primers was determined using a dilution series of a pool of cDNAs. Additional controls included the omission of reverse transcriptase to measure the extent of residual genomic DNA contamination and omission of template. Genes encoding elongation factor 1-alpha and ATP-synthase subunit beta, the transcript level of which was confirmed by means of geNorm software (http://genorm.cmgg.be/) to be stable under the applied experimental conditions (data not shown) were used for normalization of expression of the target *LOX2* gene. Three replicates were performed for each reaction. Relative expression levels were determined as the ratios between the quantities of cDNA corresponding to the target genes and values of normalization factor, which was calculated using geNorm software. The presented data were obtained by the analysis of at least ten biological replicates. ggplot2 package was used for the visualization of the expression level of *LOX2* gene.

Microscopy and immunocytochemistry

Stem sections designated in Fig. 1 of control or infected (2 days after inoculation) by *Pba* or its *cfa* mutant tobacco plants were fixed in a mixture of 2% paraformaldehyde and 0.5% glutaraldehyde prepared in 0.1 M phosphate buffer (pH 7.2) for 4 h at 4 °C, then dehydrated in a graded aqueous ethanol series, progressively infiltrated with LR White resin (Medium Grade Acrylic Resin; Ted Pella, Redding) and after that embedded in 100% resin in Beem capsules. The resin was polymerized at 60 °C for 24 h. Semi-thin sections (1 μm thick) were prepared using a glass knife on a LKB 8800 ultramicrotome (LKB Instruments). The sections were stained with 0.5% (*w*/*v*) toluidine and examined using a laser confocal microscope (LSM 510 Meta; Carl Zeiss) under transmitted light. The images were acquired with an AxioCam HRs camera (Carl Zeiss).

The immunohistochemical detection of rhamnogalacturonan I was performed using antibodies INRA-RU2 (Ralet et al. 2010) as described previously (Gorshkov et al. 2016). Briefly, the sections collected on silane-coated microscope slides were (1) pre-incubated in Na-phosphate buffered saline (PBS), pH 7.4, containing 3% (*w*/*v*) bovine serum albumin (BSA) for 1 h to block non-specific labelling; (2) incubated with the primary monoclonal antibodies INRA-RU2 diluted 1:3 in 0.1 M PBS with 0.06% (w/v) BSA; and (3) incubated with secondary goat anti-mouse antibody linked to fluorescein isothiocyanate (FITC, Sigma-Aldrich) diluted 1:100 in 0.1 M PBS with 0.06% (*w*/*v*) BSA. Primary antibodies were omitted in control experiments. The sections were examined using a laser confocal fluorescence microscope (LSM 510 Meta; Carl Zeiss). Immunofluorescence was observed using excitation at 488 nm and emission at 503–550 nm. The transmitted light channel was used for detection of anatomical details.

Statistical analysis

Determination of CFU titre and qPCR analysis were performed at least in 10 biological replicates. Statistical significance of differences among the results of each test and the relative control values was assessed using Wilcoxon signed rank, with $P < 0.05$ cutoff. The microscopy and immunocytochemistry data comprise the reproducible results of two independent experiments, each with three biological replicates.

Results and discussion

RNA-Seq data analysis

RNA sequencing of three types of samples (asymptomatic zone 1 of *Pba*-infected plant, symptomatic zone 2 of *Pba*-infected plant, and *Pba* cells cultured in vitro) was performed to compare transcriptome profiles of *Pba* at

two stages of systemic plant colonization and *Pba* cultured in vitro. After quality control and filtering, reads were aligned along the *Pba* coding sequences (Table 1).

Differentially expressed genes (DEGs) (FDR < 0.05, Log2FC > |1|) were revealed for all three possible comparisons: 1) asymptomatic zone 1 vs. symptomatic zone 2, 2) asymptomatic zone 1 vs. in vitro, 3) symptomatic zone 2 vs. in vitro (Table 2, Table S1). To get a better idea of the physiological parameters that distinguish *Pba* cells at different stages of systemic plant colonization and to identify which processes are differentially regulated *in planta* compared to in vitro conditions, the revealed DEGs were automatically classified according to KEGG pathways and modules. Overrepresentation analysis using Fisher exact test was performed in order to identify KEGG pathways and modules significantly enriched with either up- or down-regulated genes (Table 2, Table S2). Although KEGG database enables "visualization" of a number of functional gene categories (predominantly related to general metabolism), many physiological features of *Pba* cells in terms of gene expression remain "invisible" when applying this database only. Therefore, several alternative approaches for functional gene classification (both automatic and manual) were applied in our study.

Since the genes encoding proteins involved in the same biological process are often arranged in operons (Osbourn and Field 2009), classification of DEGs was performed according to DOOR2 operon database. Median and average expression levels of DEGs within operons were calculated and visualized as box plots (Fig. 2, Fig. S1, S2, Table S3). In terms of operon-located genes expression at two stages of plant colonization (asymptomatic zone 1 and symptomatic zone 2), operon No. 75611 was the most varied in transcript levels (Fig. 2). This operon contains genes coding for the enzymes necessary for coronafacic acid biosynthesis. These genes were highly up-regulated in asymptomatic zone 1 compared to symptomatic zone 2. For data regarding other differentially regulated operons refer to supplementary Table 3 and supplementary Figs. 1 and 2.

DEGs classification was performed according to CAZy, DBD, UniProt databases as well as using the data of *Pba* horizontally acquired islands (HAIs) and secretion systems published by Bell et al. (2004) and Liu et al. (2008). Merged results characterizing a pattern of *Pba* traits under *in planta* conditions drawn using the above described approaches are discussed below.

Pba genes expressed differentially *in planta* compared to in vitro conditions and at different stages of systemic plant colonization

Virulence genes

Plant cell wall degrading enzymes (PCWDEs)

The list of *Pba* genes encoding PCWDEs was classified according to CAZy database and subdivided into categories depending on the annotation of the enzymes: homogalacturonan (HG)-degrading, rhamnogalacturonan (RG)-degrading, non-pectic PCW polysaccharide (cellulose, cross-linking glycans)-degrading (Table 3, Table S4). Most of HG-degradation-related genes (11 of 16) were up-regulated *in planta* compared to in vitro conditions. Herewith, the only gene of this category (encoding periplasmic pectate lyase) was differentially expressed in two zones *in planta*. In turn, 5 of 8 genes related to RG-degradation were expressed in asymptomatic zone 1 at lower level compared to symptomatic zone 2. Two of the remaining three RG-degradation-associated genes (ECA3559 and ECA4303) were up-regulated in symptomatic zone 2 compared to in vitro conditions and non-differentially expressed in asymptomatic zone 1 compared to in vitro conditions (Table 3). This points to the fact that *Pba* behavior during two distinct stages of systemic host colonization does not differ in terms of the production of HG-related enzymes but differs with the respect to synthesis of RG-degrading enzymes. Six genes encoding the enzymes for the degradation of non-pectic PCW polysaccharides were non-differentially expressed in two analyzed zones of infected plants. Four of these genes were slightly up-regulated in symptomatic zone 2 compared to in vitro conditions and two of them were also up-regulated in asymptomatic zone 1 compared to in vitro conditions (Table 3). Further information on the expression of PCW-related genes of *Pba* under *in planta* conditions can be found in supplementary Table 4.

Observed expression levels of *Pba* PCW-degrading enzyme genes, which are considered to be the main *Pectobacterium* virulence factors, lead us to the following hypothesis. Firstly, the *Pba* brute force strategy at the advanced stage of the host colonization (symptomatic zone 2) compared to significantly stealthier behavior in asymptomatic zone 1 is not the result of up-regulation of genes coding for HG-degrading enzymes. Stealth behavior in asymptomatic zone 1 is evidently defined by lower population density (median value

Table 1 Analysis summary of the obtained RNA-Seq reads. Samples were collected from asymptomatic zone 1 and symptomatic zone 2 of *P. atrosepticum* (*Pba*)-infected plants (zones 1 and 2 are designated in Fig. 1) as well as from *Pba* cells cultured in vitro; rep. 1 and 2 mark the biological replicate

Sample	Number of reads		
	Total reads	Filtered reads	*Pba* CDS corresponding reads
zone 1 rep. 1	64,878,488	62,252,836	2,297,466 (3.69%)
zone 1 rep. 2	55,114,660	54,097,805	1,973,561 (3.65%)
zone 2 rep. 1	15,385,304	14,531,311	8,435,706 (58.05%)
zone 2 rep. 2	20,383,107	18,746,652	8,974,650 (47.87%)
In vitro rep. 1	14,819,067	14,330,666	9,415,234 (65.70%)
In vitro rep. 2	10,130,405	9,855,186	6,316,579 (64.09%)

The percentage of reads aligned to *Pba* CDS out of the total number of filtered reads is given in brackets

9.5×10^6 CFU/g) compared to necrotic zone 1 (median value 8.4×10^8 CFU/g). However, although this stealth level of bacterial density is almost two logs less than the brute force one, it is still likely to be above the quorum level. The cell titre of more than 10^6 CFU/ml was previously shown to be sufficient for *Pba* to implement cell to cell communication (Gorshkov et al. 2010). This fact is further supported by the absence of changes in the expression levels of the majority of quorum sensing-regulated genes in asymptomatic zone 1 and symptomatic zone 2. Hence, the *Pba* cell quantity in zone 1 is likely to be sufficient to form structured functional population without further increase of cell density. It is tempting to speculate that latent pectobacteria-caused infections known to frequently occur (Lapwood and Harris 1982; Helias et al. 2000; Perombelon 2002) are the result of such pathogen's population density stabilization. In this case, plant-microbial interactions seem to stay in "equilibrated" state without significant plant host fitness cost or disease symptoms development.

Secondly, *Pba* seem to degrade RG weaker within the asymptomatic zone 1 than in symptomatic zone 2. Although three genes coding for RG-degrading enzymes were up-regulated in asymptomatic zone 1 compared to in vitro conditions, most of the genes related to RG degradation were expressed at higher level in symptomatic zone 2 compared to asymptomatic zone 1. This can be explained by the requirement of this polymer for bacterial emboli initial matrix formation (Gorshkov et al. 2016). As was mentioned before, RG within the bacterial emboli matrix is being substituted by *Pba* exopolysaccharides during colonization (Gorshkov et al. 2017a); hence, RG is degraded by *Pba* more strongly at the advanced stage of colonization than at the initial one.

Table 2 Number of genes /KEGG pathways/ KEGG modules differentially regulated in *P. atrosepticum* at different stages of plant colonization (asymptomatic zone 1 and symptomatic zone 2) as well as at different colonization stages compared to in vitro conditions. Zones 1 and 2 are designated in Fig. 1

Comparison	Number of regulated genes/pathways/modules		
	zone 1 vs. zone 2	zone 1 vs. in vitro	zone 2 vs. in vitro
Up-regulated genes	47	855	1001
Down-regulated genes	168	721	688
KEGG pathways enriched with up-regulated genes	3	15	19
KEGG pathways enriched with down-regulated genes	7	8	7
KEGG modules enriched with up-regulated genes	4	16	22
KEGG modules enriched with down-regulated genes	12	16	15

Differentially expressed genes were selected according to criteria of FDR < 0.05, Log2FC > |1|; differentially regulated pathways and modules were identified using Fisher exact test ($P < 0.05$)

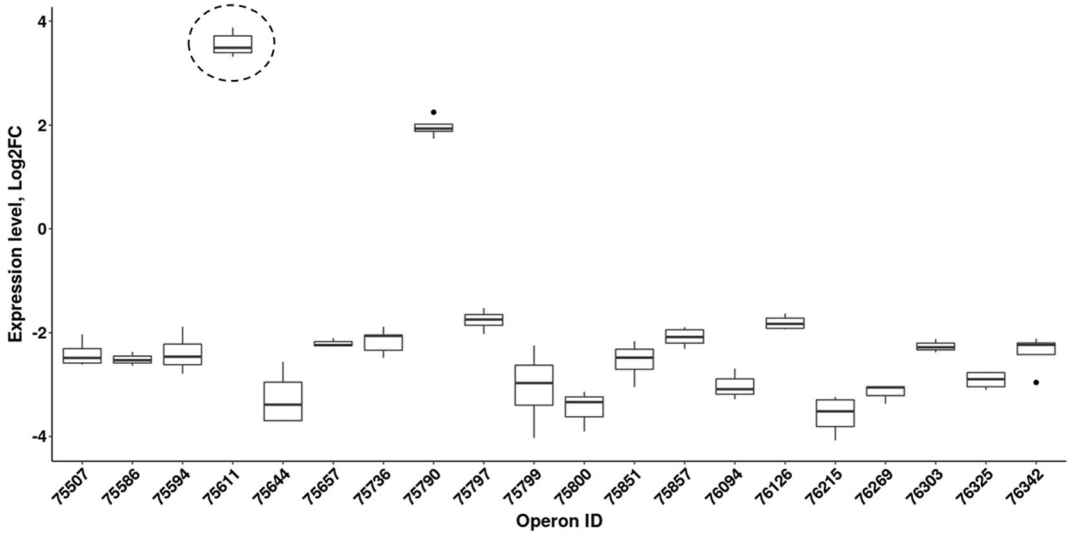


Fig. 2 Transcript levels of *P. atrosepticum* operon located genes (according to DOOR2 database), which show different expression at different stages of systemic plant colonization (asymptomatic zone 1 vs. symptomatic zone 2). Zones 1 and 2 are designated in Fig. 1. Operon No. 75611 harboring genes for coronafacic acid biosynthesis is denoted by the dotted circle. Other data on differentially regulated operons is present in supplementary Table 3

Secretion systems

Pba genes encoding components of different secretion systems types were selected based on classification previously performed (Bell et al. 2004; Liu et al. 2008 and UniProt annotations, Fig. 3, Table S5). Five gene clusters from *Pba* genome related to T1SS were selected for analysis (T1SS_1 – ECA1096–1098, T1SS_2 – ECA1534–1538, T1SS_3 – ECA0800–0803, T1SS_4 – ECA2781–2785, T1SS_5 – ECA3266–3269). Four of them (T1SS_2-T1SS_5) were expressed differentially *in planta* compared to in vitro conditions (Fig. 3, Table S5). T1SS_2 is presumably involved in siderophore transport. ECA1534–1536 operon (operon DOOR2 ID 75800) and ECA1538 ORF display high level of similarity with a canonical *has*-cluster encoding components of siderophore-transporting machinery in *Serratia marcescence*, *Pseudomonas aeruginosa*, *Vibrio cholerae*, *Neisseria meningitis*, *E. coli* (Thomas et al. 2014). Additionally, ECA1537 encodes a heme-binding protein that may be involved in siderophore capture. All the genes of *Pba has*-cluster were highly induced at advanced stage of colonization (symptomatic zone 2) compared to in vitro conditions and also up-regulated compared to asymptomatic zone 1 pointing to the role of T1SS_2 in determination of aggressive behavior of *Pba*. T1SS_3 which functions remain unknown was also induced in symptomatic zone 2 but not in asymptomatic zone 1 (Fig. 3, operon DOOR2 ID 75647, Table S5).

All the genes of T1SS_4 (operon DOOR2 ID 76019) in *Pba* having high similarity with metalloprotease exporting T1SS of *Erwinia chrysanthemi* (Song et al. 2006) were up-regulated in asymptomatic zone 1 and most of them were induced in symptomatic zone 2 compared to in vitro conditions. This indicates that metalloproteases might play significant role at both stages of systemic colonization (Fig. 3, Table S5). In contrast, T1SS_5 essential for the secretion of a proteinaceous multi-repeat adhesin (MRP) as well as the regulators of this system encoded by ECA3265, ECA3270, ECA3271 (Pérez-Mendoza et al. 2011) were down-regulated at both stages of systemic colonization compared to in vitro conditions (Fig. 3, Table S5). MRP was shown to provide adhesion of *Pba* cells to root surface (Pérez-Mendoza et al. 2011); therefore, T1SS_5 seems to be required during invasion stage of pathogenesis. However, after host invasion, during the systemic colonization of the plant tissues, this system is unlikely to be important as indicated by the down-regulation of T1SS_5-related genes in zones 1 and 2 compared to in vitro conditions.

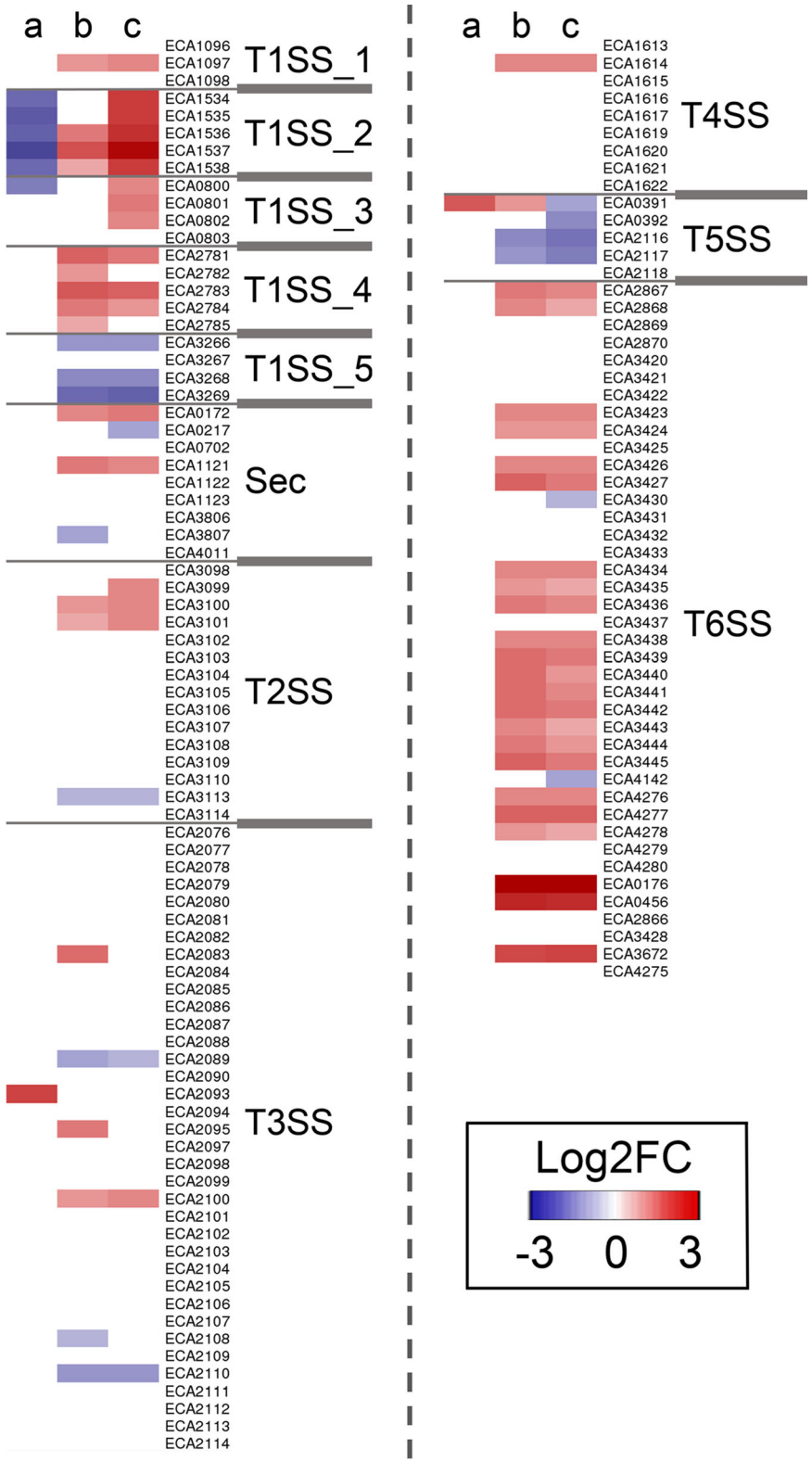
Surprisingly, most of the genes encoding components of Sec-pathway and T2SS, which are required for PCWDEs translocation were non-differentially expressed under *in planta* and in vitro conditions (Fig. 3, Table S5). However, the absolute expression level of these genes (in terms of counts of reads per transcript) was high. This can indicate that T2SS is required under

Table 3 Expression levels of *P. atrosepticum* genes encoding plant cell wall degrading enzymes (PCWDEs). The list of PCWDE genes was built and subdivided into several categories according to CAZy database and UniProt annotation of the enzymes: homogalacturonan (HG)-degrading, rhamnogalacturonan (RG)-degrading, non-pectic PCW polysaccharide-degrading. The relative expression levels were determined under *in planta* conditions in asymptomatic zone 1 compared to symptomatic zone 2 as well as in zone 1 compared to in vitro culture and in zone 2 compared to in vitro culture. Zones 1 and 2 are designated in Fig. 1. Red and blue color are assigned to up- and down-regulated genes, respectively. More data relating to the expression of PCW-related genes of *Pba* under *in planta* conditions can be found in supplementary Table 4

Locus tag	CAZy family	CAZy annotation	UniProt annotation	Expression level (Log2FC)		
				Zone 1 vs. Zone 2	Zone 1 vs. *in vitro*	Zone 2 vs. *in vitro*
Homogalacturonan-degrading						
ECA3552	GH28	polygalacturonase	putative polygalacturonase	nonDEG	nonDEG	nonDEG
ECA1095	GH28	polygalacturonase	endo-polygalacturonase	nonDEG	-1,2	-1,4
ECA1190	GH28	polygalacturonase	putative polygalacturonase	nonDEG	2,4	3,1
ECA3111	GH28	polygalacturonase	exo-poly-galacturonosidase	nonDEG	nonDEG	nonDEG
ECA4067	PL1	pectate lyase	pectate lyase I	nonDEG	1,2	2,2
ECA4068	PL1	pectate lyase	pectate lyase II	nonDEG	nonDEG	nonDEG
ECA4069	PL1	pectate lyase	pectate lyase III	nonDEG	3,1	3,5
ECA4070	PL1	pectate lyase	pectate lyase	nonDEG	2,6	2,0
ECA3112	PL1	pectate lyase	pectate lyase	nonDEG	1,5	2,5
ECA1499	PL1	pectate lyase	pectin lyase	nonDEG	1,1	1,8
ECA2402	PL2	pectate lyase	pectate disaccharide-lyase	nonDEG	1,1	2,2
ECA2135	PL2	pectate lyase	periplasmic pectate lyase	1,8	nonDEG	-1,7
ECA1094	PL3	pectate lyase	pectate lyase	nonDEG	nonDEG	1,9
ECA4510	PL9	pectate lyase	exopolygalacturonate lyase	nonDEG	2,2	3,2
ECA2553	PL9	pectate lyase	pectate lyase	nonDEG	1,6	1,6
ECA2426	PL22	oligogalacturonate lyase	oligogalacturonate lyase	nonDEG	2,6	3,9
Rhamnogalacturonan-degrading						
ECA3559	GH105	rhamnogalacturonyl hydrolase	plant-inducible protein	nonDEG	nonDEG	2,8
ECA3749	GH105	rhamnogalacturonyl hydrolase	plant-inducible protein	nonDEG	4,0	5,1
ECA0852	GH53	endo-β-1,4-galactanase	putative proteoglycan hydrolase	-2,3	nonDEG	2,1
ECA3178	GH53	endo-β-1,4-galactanase	endo-1,4-beta-galactosidase	-3,3	1,3	4,6
ECA1490	GH2	β-galactosidase	beta-galactosidase	-2,8	3,4	6,3
ECA3179	GH42	β-galactosidase	beta-galactosidase	-2,4	nonDEG	3,1
ECA4303	PL26	rhamnogalacturonan exolyase	putative exported protein	nonDEG	nonDEG	1,8
ECA0804	PL4	rhamnogalacturonan exolyase	rhamnogalacturonate lyase	-2,1	nonDEG	2,5
Non-pectic PCW polysaccharide-degrading						
ECA3793	GH43	β-xylosidase	putatuve glycosysl hydrolase	nonDEG	nonDEG	1,1
ECA3847	GH43	β-xylosidase	putative xylosidase/arabinosidase	nonDEG	-1,1	nonDEG
ECA2220	CBM63	CBM63 module of expansin EXLX1	putative cellulase	nonDEG	3,1	1,7
ECA2827	GH12	endoglucanase	beta(1,4)-glucan glucanohydrolase	nonDEG	nonDEG	nonDEG
ECA4373	GH8	chitosanase	endoglucanase	nonDEG	nonDEG	1,5
ECA1981	GH5	endo-β-1,4-glucanase	endoglucanase V	nonDEG	2,1	1,4

both *in planta* and in vitro conditions. This is further supported by the following facts. Firstly, bacterial species that do not live inside the host body also have T2SS. Secondly, many proteins other than PCWDEs, which may be required "outside" the host are secreted via T2SS (Cianciotto and White 2017). Therefore, it is possible that even though T2SS-secreted proteins are produced differentially under different environmental conditions, the core components of T2SS are synthesized more or less constitutively. In our study, most of the genes encoding T2SS-secreted PCWDEs were up-regulated *in planta* compared to in vitro condition (see above). In addition, genes of two other T2SS-secreted *Pba* virulence factors – Nip (ECA3087) and Svx (ECA0931) (Mattinen et al. 2004; Corbett et al. 2005; Pemberton et al. 2005; Coulthurst et al. 2008) were highly induced at both stages of systemic plant colonization (Table S1, S7).

Most of *Pba* type III secretion system (T3SS) related genes (ECA2076–2114, Bell et al. 2004, UniProt) were


a b c
ECA1096
ECA1097
ECA1098
T1SS_1
ECA1534
ECA1535
ECA1536
ECA1537
ECA1538
T1SS_2
ECA0800
ECA0801
ECA0802
T1SS_3
ECA0803
ECA2781
ECA2782
ECA2783
ECA2784
ECA2785
T1SS_4
ECA3266
ECA3267
ECA3268
ECA3269
T1SS_5
ECA0172
ECA0217
ECA0702
ECA1121
ECA1122
ECA1123
ECA3806
ECA3807
ECA4011
Sec
ECA3098
ECA3099
ECA3100
ECA3101
ECA3102
ECA3103
ECA3104
ECA3105
ECA3106
ECA3107
ECA3108
ECA3109
ECA3110
ECA3113
ECA3114
T2SS
ECA2076
ECA2077
ECA2078
ECA2079
ECA2080
ECA2081
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ECA2108
ECA2109
ECA2110
ECA2111
ECA2112
ECA2113
ECA2114
T3SS
a b c
ECA1613
ECA1614
ECA1615
ECA1616
ECA1617
ECA1619
ECA1620
ECA1621
ECA1622
T4SS
ECA0391
ECA0392
ECA2116
ECA2117
ECA2118
T5SS
ECA2867
ECA2868
ECA2869
ECA2870
ECA3420
ECA3421
ECA3422
ECA3423
ECA3424
ECA3425
ECA3426
ECA3427
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ECA3441
ECA3442
ECA3443
ECA3444
ECA3445
ECA4142
ECA4276
ECA4277
ECA4278
ECA4279
ECA4280
ECA0176
ECA0456
ECA2866
ECA3428
ECA3672
ECA4275
T6SS
Log2FC
-3 0 3

Fig. 3 Heatmap represents the relative expression levels of genes encoding proteins associated with different secretion system types (marked on the right side of the heatmap) of *P. atrosepticum*. **a** – the expression level *in planta* in asymptomatic zone 1 compared to symptomatic zone 2; **b** – in asymptomatic zone 1 compared to in vitro conditions; **c** – in symptomatic zone 2 compared to in vitro conditions. Zones 1 and 2 are designated in Fig. 1. Color scale corresponds to the values (Log2FC) of relative expression levels (red – up-regulated; blue – down-regulated). Additional information on the expression of secretion system-related genes of *Pba* under *in planta* conditions is present in supplementary Table 5

non-differentially expressed under in *planta* and in vitro conditions (Fig. 3, Table S5); moreover, expression levels of these genes were very low. This shows that T3SS is unnecessary for the systemic plant colonization. However, this system is unlikely to be rudimentary, since the reduced virulence of T3SS-mutants of pectobacteria was demonstrated (Ageichik et al. 2002; Holeva et al. 2004). Presumably, the role of this system in *Pba* is restricted to invasive stage of interaction with the host plant. Genes of *Pba* T4SS (T4SS–ECA1613–1622, Bell et al. 2004) were non-differentially expressed under *in planta* and in vitro conditions (Fig. 3, Table S5).

Genes related to two partner secretion system (TPSS) that represents a branch of T5SS, in *Pba* are located within two clusters (TPSS_1–ECA0391–0392, TPSS_2–ECA2116–2118 (*hecA1*, *hecA2*, *hecB*), Bell et al. 2004). In plant pathogenic bacteria, TPSS is involved in the secretion of high molecular weight filamentous hemagglutinin (FHA)-like adhesins (HecA) that provide cell adhesion and biofilm formation (Guérin et al. 2017). HecA-mutants of *Erwinia chrysanthemi* (*Dickeya sp.*) related to *Pba* showed reduced ability to adhere to plant tissues (Rojas et al. 2002). Although, in many instances, the TPSS operons are induced upon bacterial entry into the host (Guérin et al. 2017), in *Pba* most of TPSS-related genes were down-regulated *in planta* compared to in vitro conditions (Fig. 3, Table S5). TPSS may be efficient for getting into the host from the environment that is supported by its necessity for *E. chrysanthemi* to adhere to plant tissues, but unnecessary for systemic plant colonization.

T6SS-related genes in *Pba* described by Liu et al. (2008) include those arranged within a cluster (ECA3420–3445) and diffusely located ones that encode T6SS-exported proteins HcpA detected in *Pba* secretome (Mattinen et al. 2007). Most of the T6SS-related genes (also represented in KEGG module eca_M00334 "Type VI secretion system"; operon DOOR2 IDs 76150, 76151) including 4 of 7 HcpA-encoding ones were up-regulated at both stages of plant colonization compared to in vitro conditions (Fig. 3, Table S5). As indicated by the expression level, this system is likely to be necessary at both analyzed stages of plant colonization. These facts are consistent with hypervirulence of *hcpA*-overexpressing *Pba* strain (Mattinen et al. 2007) and reduced virulence of T6SS-defective *Pba* mutant (Liu et al. 2008).

Taken together, some secretion systems are regulated in *Pba* during systemic plant colonization. T1SS_2 presumably involved in the transport of siderophores is regulated differentially at different stages of colonization with up-regulation only during advanced rot-associated stage (symptomatic zone 2); therefore, this system may presumably be a marker for the brute force behavior of *Pba*. Both analyzed stages of plant-*Pba* interactions are characterized by the down-regulation of genes of those secretion systems that are involved in cell adhesion (T1SS_5, T5SS). This is in accordance with the absence of adhesion of *Pba* cells to PCW in the course of systemic plant colonization reported in our previous studies (Gorshkov et al. 2014, 2016). T3SS, which is a well-known device associated with stealth biothrophic pathogens, is necessary for manipulation of plant cell responses. RNA-Seq analysis of *Pba* did not show importance of T3SS during analyzed stages of interaction with the plant. In contrast, another stealth behavior-associated secretion system (T6SS) that contributes to *Pba* virulence (Liu et al. 2008) was induced in *Pba in planta* compared to in vitro conditions. This supports a previous suggestion that pectobacteria, such as *Pba*, in addition to brute force strategy may also employ stealth strategy when interacting with plants in certain conditions (Toth and Birch 2005; Liu et al. 2008; Gorshkov et al. 2016).

Motility

Over two thirds of *Pba* genes related to flagellar assembly and chemotaxis (including KEGG pathways eca02030 "Bacterial chemotaxis" and eca02040 "Flagellar assembly"; operon DOOR2 ID 75824, 75828, 75831) were down-regulated *in planta* in both asymptomatic zone 1 and symptomatic zone 2 compared to in vitro conditions (Table S6). These results are in agreement with the previously shown negative effect of plant extract on the expression of motility-

associated genes in *Pba* (Mattinen et al. 2008). On one hand, reduction of flagella synthesis inside the host is a reasonable way to avoid activation of defense reactions, since bacterial flagellin is a well-known PAMP (pathogen-associated molecular pattern) that induces immune responses in the host. Furthermore, pathogenic bacteria, including a phytopathogenic *P. syringae* were shown to down-regulate flagellar apparatus after reaching the proper host site (Soutourina and Bertin 2003; Yu et al. 2012; Chaban et al. 2015). In addition to attenuation of host defenses, such kind of strategy also allows to reduce energy costs for the maintenance of flagellar apparatus.

On the other hand, *in planta* down-regulation of flagella/chemotaxis-related genes contradicts the observed active movement of *Pba* inside the plant and downward migration through the xylem vessels in particular (Gorshkov et al. 2014). Such kind of inconsistency may be explained by the following. First, the quantitative ratio of motile/non-motile cells in bacterial population was shown to vary depending on the environmental conditions (Stewart and Cookson 2012; Koirala et al. 2014). Therefore, only individual *Pba* cells may implement active movement in the course of systemic plant colonization and a greater portion of cells may realize sedentary lifestyle. Due to that, possible activation of expression of motility-related genes in individual cells cannot be detected by RNA-Seq analysis, since the transcripts from a pool of cells are analyzed. Second, the bacterial motility may be implemented not only by means of flagellar apparatus (swimming, swarming), but also by pili/fimbriae (twitching) (Mattick 2002). For *P. carotovorum* ssp. *brasiliense*, pili/fimbriae-associated motility was shown to take place *in planta*; herewith, when the activity of flagellar apparatus (but not pili/fimbriae) was disturbed by quorum sensing-related mutation, the bacteria were still able to colonize systemically their host plant (Moleleki et al. 2017).

In *Pba*, three clusters of genes (T4P_1–ECA0787–0794, T4P_2– ECA0532- ECA0545, T4P_3–ECA1667–1669) encode proteins that share similarity with type IV pili (T4P) components (Table S6). All the genes of T4P_1 cluster were highly induced in symptomatic zone 2 (operon DOOR2 ID 75644). This cluster in *Pba* was previously shown to encode Flp/Tad pili (attributed to type IVb pili) that play significant role in disease development (Nykyri et al. 2013). In contrast to type IVa pili, type IVb pili were not experimentally shown to retract, but were proposed to implement retraction-like activity that may provide the cell motion (Burrows 2012). All these facts suggest that systemic spread of pectobacteria within the plants is carried out, at least to some extend, in flagella-independent manner that is consistent with the role of twitching motility and T4P in downward translocation of some phytopathogens through the xylem vessels (Meng et al. 2005; Wairuri et al. 2012). Additionally, T4P_1 in *Pba* was down-regulated in asymptomatic zone 1 compared to symptomatic zone 2 pointing to differential role of Flp/Tad pili at different stages of plant colonization.

Other virulence-related genes

The most striking difference for two analyzed stages of colonization in terms of *Pba* gene expression has been observed for the genes encoding enzymes responsible for coronafacic acid (Cfa) biosynthesis. This compound is well known as one of two components of *Pseudomonas syringae* phytotoxin coronatine (Bender 1999). Although genes for the second component, coronamic acid, synthesis are absent in *Pba* genome, Cfa itself was shown to contribute to *Pba* virulence (Bell et al. 2004; Toth et al. 2006). Only 3 of 11 genes of *Pba cfa*-operon were slightly induced (log2 FC < 2) in the symptomatic zone 2 (Table S7) while, the whole *cfa*-operon (DOOR2 ID 75611) as well as a predicted LysR-type regulator were significantly up-regulated in asymptomatic zone 1 compared to both symptomatic zone 2 and in vitro conditions (Fig. 4, Table S7).

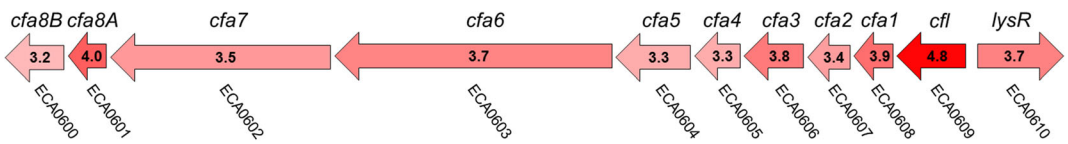

Fig. 4 Scheme *P. atrosepticum cfa*-cluster (*cfa*-operon No. 75611, DOOR2 database and a predicted LysR-type regulator). Numbers in arrows are gene expression level values (Log2FC) in asymptomatic zone 1 compared to symptomatic zone 2 *in planta*. Zones 1 and 2 are designated in Fig. 1

In contrast to *cfa*-cluster, genes related to phosphonate metabolism (KEGG pathway eca00440 "Phosphonate and phosphinate metabolism") were expressed at significantly higher levels in symptomatic zone 2 compared to asymptomatic zone 1. Also these genes were induced in both zones *in planta* compared to in vitro conditions (operon DOOR2 ID 75594, Table S7). Phosphonates are highly diverse group of compounds that contain C-P (carbon-phosphorus) bond. Phosphonate moieties may be found in macromolecules, such as carbohydrates and lipids, and are predicted to influence their properties. These compounds may also serve as reservoir of the phosphorus necessary for the cellular metabolism. In addition, certain phosphonates are low molecular weight compounds, some of which were shown to have physiological activity and herbicidal properties (Horsman and Zechel 2016). In *Pba*, genes for phosphonate biosynthesis and C-P lyase degradative pathway are arranged in one cluster (ECA0487–0498). To date, phosphonates of pectobacteria as well as other phytopathogenic bacteria were not studied well, therefore, role of up-regulation of ECA0487–0498 cluster *in planta* is enigmatic. However, one can speculate that *Pba* phosphonates contribute to virulence and possess phytotoxic properties. In addition to up-regulation of phosphonate-related cluster during plant colonization, the induction of *Pba* genes encoding non-ribosomal peptide synthases (KEGG pathway eca01053 "Biosynthesis of siderophore group nonribosomal peptides") *in planta* reinforces this assumption, since natural phosphonate herbicides are synthesized with the help of these enzymes (Blodgett et al. 2005; Schwartz et al. 2005). Besides, genes for one of the group of *in planta* up-regulated non-ribosomal peptide synthases in *Pba* are located in close proximity (ECA0482–0483) to phosphonate-related cluster (ECA0487–0498). Additionally, phosphonate-related cluster contains a gene (ECA0494, which is also up-regulated *in planta*) encoding phosphonate ABC-transporter that may be involved in translocation of these compounds.

Non-ribosomal peptide synthases are also involved in siderophore synthesis. These are high-affinity iron-chelating compounds secreted by microorganisms to scavenge iron from their environment including host plant interior. Siderophores were demonstrated to contribute to plant pathogenic bacteria virulence (Franza et al. 2005; Dellagi et al. 2005, 2009; Burbank et al. 2015). In our study, RNA-Seq analysis revealed that many genes associated with synthesis and transport of siderephores (operon DOOR2 ID 75593) and iron transport (including KEGG module eca_M00240 "Iron complex transport system"; operon DOOR2 ID 75706, 75792) were up-regulated *in planta* compared to in vitro conditions; herewith, only tiny portion of siderophore/iron-related genes were expressed differentially in asymptomatic zone 1 compared to symptomatic zone 2 pointing to a significant role of iron assimilation during both stages of plant colonization (Table S7).

Stress-related genes

Even though plant tissue is a natural environment for phytopathogens, they still suffer from a variety of stresses during host colonization, and pathogens with impaired stress response-related genes were demonstrated to be less virulent (Andersson et al. 1999; Nachin et al. 2005). 110 of the revealed DEGs encoded the proteins related to bacterial stress response; 53 of them were up-regulated *in planta* compared to in vitro conditions (Table S8). One of the main stress factors for bacteria inside the plant organism is reactive oxygen species (ROS). To cope with ROS, bacteria evolved a variety of antioxidant systems (Cabiscol Català et al. 2000). The revealed DEGs related to antioxidant systems were both up- and down-regulated. The most significant increase of expression *in planta* compared to in vitro conditions was observed for 4 genes encoding electron carriers that participate in redox metabolism (ferredoxins, glutaredoxin, flavodoxin) (ECA2663, ECA3335, ECA3336, ECA4034) (Table S8). The former three of them also showed changes in expression levels at different stages of plant colonization with down-regulation in asymptomatic zone 1 compared to symptomatic zone 2. A gene for flavohemoprotein HmpX previously shown by an example of *E. chrysanthemi* (*D. dadantii*) to act as NO-scavenger and to attenuate plant hypersensitive reaction during infection (Boccara et al. 2005) was also among the most induced genes under *in planta* conditions.

DEGs encoding stress proteins (e.g. cold/heat shock proteins, osmotically inducible proteins, carbon starvation protein) as well as those associated with antibiotics/multidrug resistance (including KEGG categories eca_M00701 "Multidrug resistance"; eca_M00721, eca_M00722, eca_M00724, eca01503 "Cationic antimicrobial peptide (CAMP) resistance"; eca00261 "Monobactam biosynthesis") and xenobiotic degradation were represented by both up- and down-regulated genes in a similar ratio (Table S8). Only 10 out of 53 up-regulated *in planta* and 12 of 57 down-regulated *in planta* stress-related genes (this study) were previously shown to be induced or

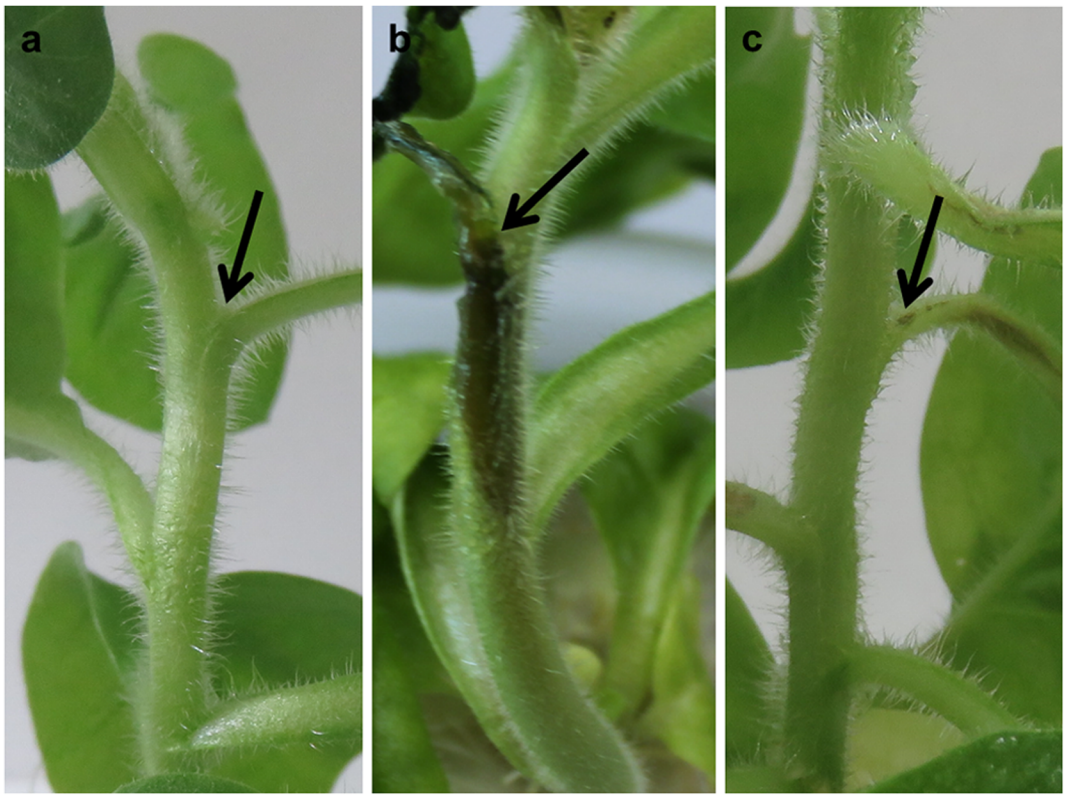

Fig. 5 Stems of non-infected tobacco plants (**a**) or infected with the wild type *P. atrosepticum* SCRI1043 (**b**) or its *cfa* derivative (**c**). Photographs were taken 2 days after plant inoculation. Arrows indicate the inoculation point of bacterial suspension or 10 mM $MgCl_2$

repressed, respectively, during stress adaptation of *Pba* to starvation (Gorshkov et al. 2017b). This shows that the strategies of bacterial stress adaptation *in* and *ex planta* are quite different that is in accordance with the existence of alternative types of stress responses chosen by *Pba* depending on its physiological state, population density and type of the stress factor (Gorshkov et al. 2010; Petrova et al. 2014, 2016). Additional information on the expression of stress-related genes of *Pba* under *in planta* conditions is present in supplementary Table 8.

The data on the expression of genes related to cell envelope, transporters, general metabolism, regulators and proteins of unknown functions are described in a text given in the supplementary file.

Role of coronafacic acid (Cfa) in systemic plant colonization by *Pba*

Since the most dramatic difference in *Pba* gene expression at two analyzed stages of colonization was observed for the genes of coronafacic acid (Cfa)-related cluster (up-regulation in asymptomatic zone 1 compared to symptomatic zone 2, Figs. 2, and 4), we have constructed Cfa biosynthesis deficient mutant of *Pba* by knocking out the *cfa6* gene and compared the strategies of plant colonization by the wild type *Pba* and its *cfa* mutant. Infection by the *cfa* mutant, in contrast to the wild type, did not lead to the "soft" macerated area formation in the stem. Instead, *cfa* mutant-inoculated plants displayed only tiny area of "dry" necrosis or did not show any disease symptoms (Fig. 5). This is in line with the reduced virulence of *cfa* mutant on potato plants compared to the wild type *Pba* (Bell et al. 2004).

The titre of the wild type bacterial cells *in planta* in symptomatic zone 2 was more than a log higher (8×10^8 CFU/g of plant material) than that of mutant cells (3×10^7 CFU/g) in the analogous part of the stem (Fig. 6). Since *Pba* is known to implement downward migration in plants through the xylem vessels (Gorshkov et al. 2014), sections 0.5 and 1 cm below macerated stem areas and sections from the same stem level of *cfa*

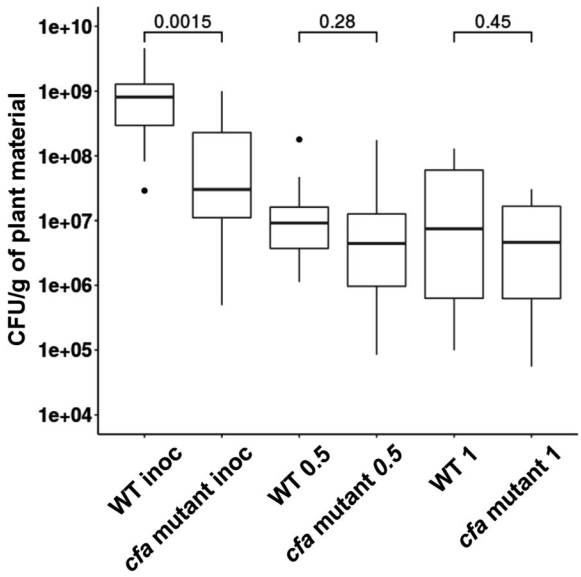

Fig. 6 Cell titre of the wild type (WT) *P. atrosepticum* or its *cfa* mutant (*cfa*) in different zones of the infected tobacco plant stems. Stem samples were taken two days after plant inoculation from inoculation zone (inoc) where in the case of the wild type bacteria extensive maceration was visible as well as 0.5 cm (0.5) and 1 cm (1) below the macerated area. The designations of analyzed plant samples are given in Fig. 1. No bacteria were recovered from control non-infected plants. The experiments were performed in at least 10 biological replicates; significance level of differences was calculated using Wilcoxon signed rank test

mutant-inoculated plants were compared in terms of bacterial cell titre. The cell density in these areas was around 1×10^7 CFU/g, and no significant differences in the titre were observed for the wild type and mutant strains (Fig. 6). This points to the absence of distinctions in initial steps of systemic plant colonization by the wild type and *cfa* mutant bacteria.

Downward migration of *Pba* is associated with the formation of bacterial emboli (Gorshkov et al. 2014). Therefore, we have performed the microscopic analysis and found that both wild type and *cfa* mutant strains were able to form bacterial emboli in the primary xylem vessels (Fig. 7 c, d, g, h). In addition, the wild type strain in symptomatic zone 2, but not in asymptomatic stem areas, heavily colonized core parenchyma, which was almost destroyed in symptomatic zone (Fig. 7 e, f). Herewith, cells of *cfa* mutant were not found in core parenchyma in both asymptomatic zone 1 and symptomatic zone 2 (Fig. 7i, j).

Bacterial emboli formation is preceded by the release of one of the pectic PCW polysacchrides – rhamnogalacturonan I (RGI) from the PCW into the vessel lumen, where this polymer forms an initial matrix for bacterial cells (Gorshkov et al. 2016). By using RGI-specific antibodies (INRA-RU2, Ralet et al. 2010) we have shown that the release of RGI into the lumen occurs during systemic colonization of plants by the *cfa* mutant to the same extent as by the wild type strain (Fig. 8). All these data show that *cfa* mutant displays the features of the wild type strain during biotrophic-like stage of systemic colonization; however, this mutant, in contrast to the wild type *Pba*, is unable to perform a further switch to the necrotrophic phase of interaction with the host plant.

In *P. syringae*, phytotoxin coronatine (consisting of Cfa and coronamic acid) promotes the induction of jasmonate-mediated pathway that is the necessary requirement for the disease progression (Uppalapati et al. 2007). Therefore, we have compared the wild type and *cfa* mutant strains in their ability to induce jasmonate-mediated pathway in plants by analyzing the expression level of the *LOX2* gene – a marker for this plant hormonal system (Ren et al. 2010). The wild type strain induced *LOX2* expression by two orders of magnitude compared to the non-infected control plants (Fig. 9). Herewith, the expression of this gene was induced only slightly (3 times) in *cfa* mutant-infected plants compared to the control ones.

Taken together, our experiments show that *cfa*-cluster is unnecessary for initial stage of systemic plant colonization (asymptomatic zone 1), when bacteria predominantly colonize xylem vessels, form bacterial emboli and do not cause maceration symptoms. However, at this particular stage, the induction of *cfa*-cluster expression occurs promoting over-induction of host plant jasmonate-mediated pathway that is required for the subsequent transition to necrotrophic stage of colonization. In turn, at necrotrophic stage, when jasmonate-mediated response is already induced, the expression of *cfa*-cluster is reduced. This indicates that jasmonate-mediated hormonal system is an attractive target to restrain pectobacteria within biotrophic phase and, thus, reduce damage caused by these phytopathogens.

General conclusions

In this study, we have revealed the genes of *P. atrosepticum* expressed differentially *in planta* compared to in vitro conditions. Furthermore, we have

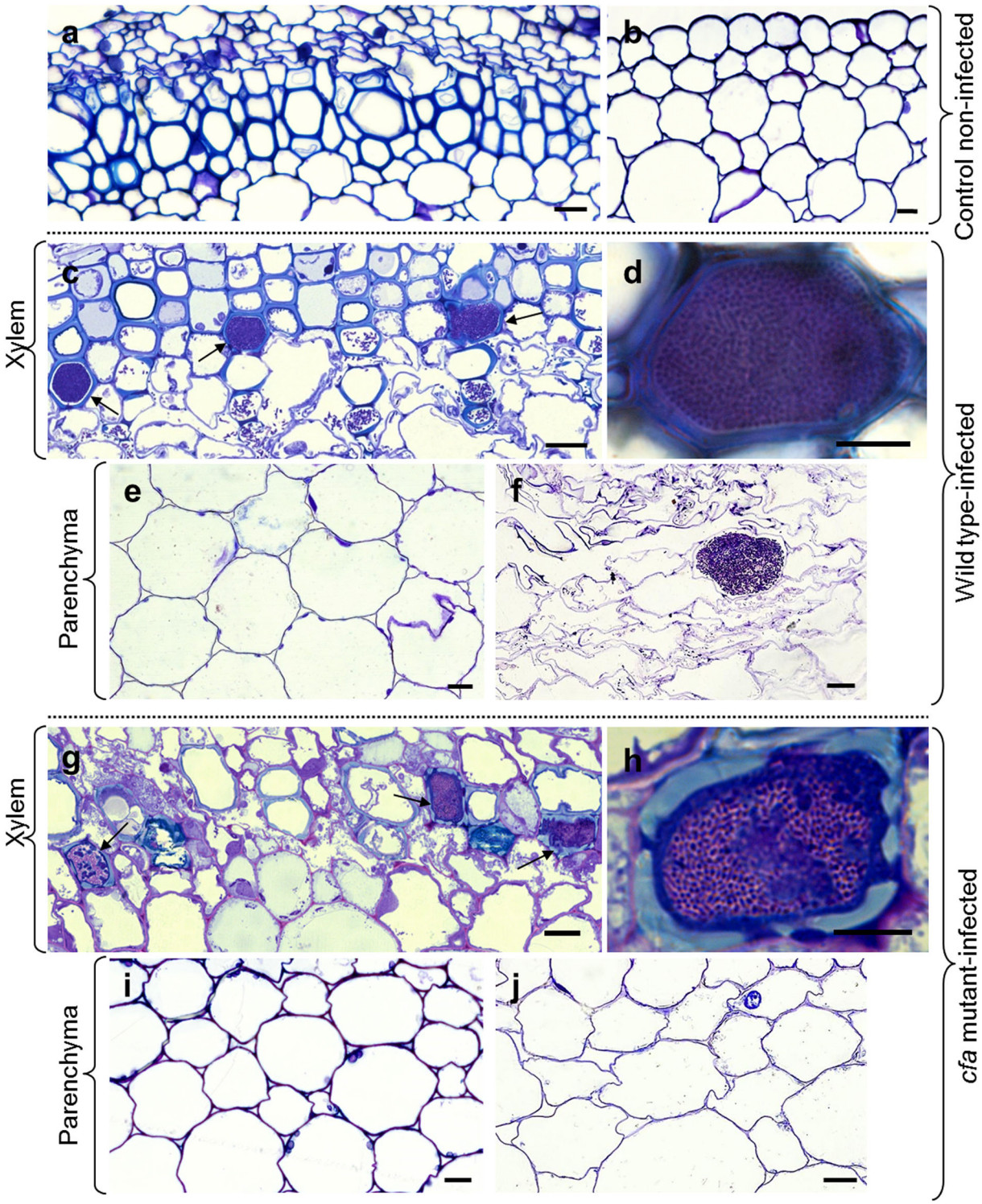

Fig. 7 Stem sections of tobacco plants non-infected (**a, b** – xylem and core parenchyma areas, respectively), or infected with either the wild type *P. atrosepticum* (**c**-**f**) or its *cfa* mutant (**g**-**j**). c and g – xylem zone with bacterial emboli (arrows); **d** and **h** – magnified images of bacterial emboli. **e**, **f**, **i**, **j** – core parenchyma areas. Stem samples were taken two days after plant inoculation from inoculation zone (**f**, **j**) where in the case of the wild type bacteria extensive maceration was visible, as well as a zone 0.5 cm below the macerated area (**c**-**e**, **g**-**i**). The designations of analyzed plant samples are given in Fig. 1. Scale bars – 20 μm (**a**-**c**, **e**-**g**, **i**, **j**), 10 μm – (**d**, **h**)

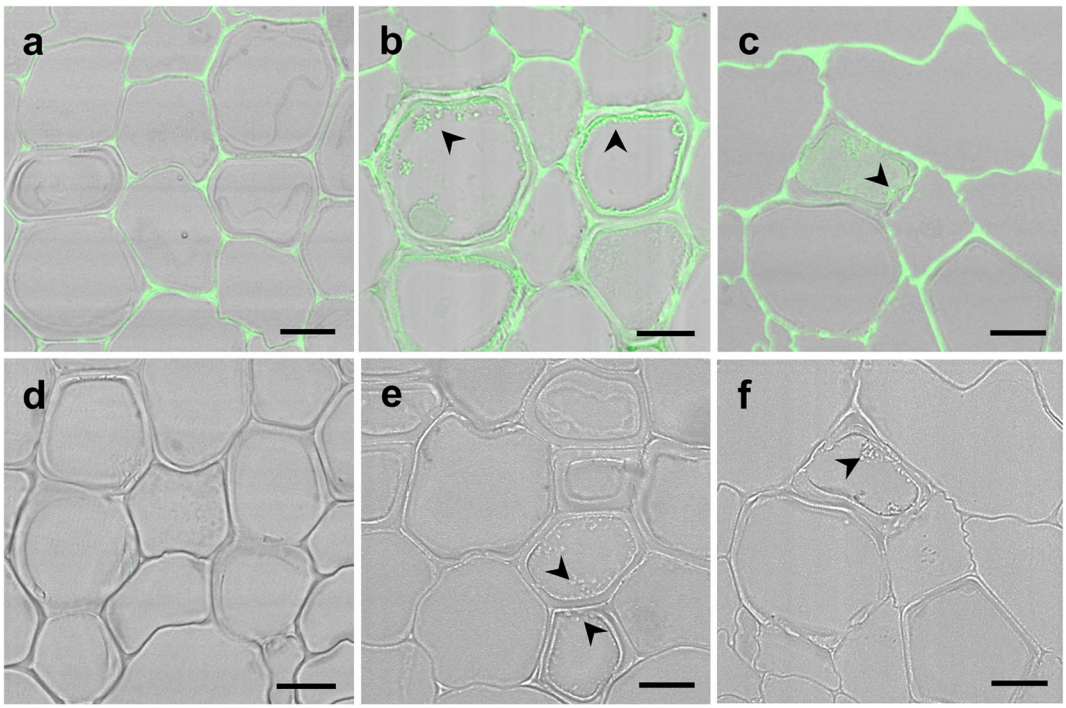

Fig. 8 Indirect immunofluorescence detection of rhamnogalacturonan I (RG-I) epitopes by INRA-RU2 antibodies (Ralet et al. 2010) on the stem sections of tobacco plants non-infected (**a**, **d**), or infected with either the wild type *P. atrosepticum* (**b**, **e**) or its *cfa* mutant (**c**, **f**). Arrowheads mark RG-I released from cell walls into the lumens of primary xylem vessels. Section (**d**-**f**) were treated with secondary but not primary, antibody. Scale bars correspond to 10 μm

identified those genes, whose transcript levels vary depending on the stage (symptomless or soft-rot

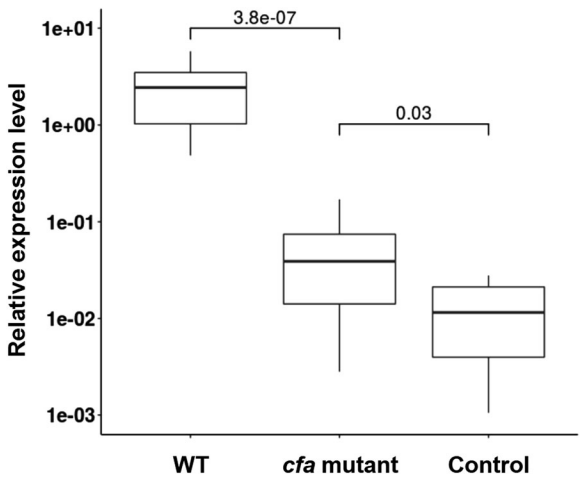

Fig. 9 The expression level of *LOX2* gene in control and infected by the wild type *P. atrosepticum* SCRI1043 (WT) or its *cfa* mutant (*cfa*) tobacco plants. The transcript level was assessed in fully expanded leaves located below the inoculation zone two days after plant infection. The experiments were performed at least in 10 biological replicates; significance level of differences was calculated using Wilcoxon signed rank test

associated) of systemic plant colonization. The data acquired, allowed us to suggest that the switch from stealth to brute force mode of *Pba* behavior in the course of systemic plant colonization is coupled with changes in their transcriptome profile. We could also identify the traits (increase in rhamnogalacturonan-degrading enzymes, up-regulation of siderophore-related T1SS, induction of Flp/Tad pili, production of Cfa, synthesis of phosphonates) predominantly associated with one of the two analyzed colonization stages. The strongest candidate identified from gene expression profiling, as required for correct switch of *Pba* behavior – Cfa – was confirmed experimentally by generating a *cfa* mutant strain and assessing its ability to colonize tobacco plants. Intact *cfa* locus appeared to be unnecessary for establishment of the asymptomatic stage of plant colonization. Strikingly, this locus seems to be required for the transition to symptomatic soft rot-associated stage coupled with over-induction of jasmonate-mediated pathway in the plant.

Interestingly, stealth and brute force behavior of *Pba* did not differ in the expression level of genes encoding major virulence factors – homogalacturonan-degrading

enzymes. Apparently, the destructive action of these enzymes during asymptomatic stage of infection was not evident due to the reduced cell titre in the asymptomatic zone compared to symptomatic one and presumably because of specific localization of bacteria *in planta*: in the primary xylem vessels but not in the core parenchyma. It should be mentioned that although *Pba* cell density in asymptomatic zone is lower than that in symptomatic one, it is above the quorum level (that is additionally supported by the up-regulation of quorum-sensing related genes) and, thus, seems to be sufficient for the transition of bacteria through the whole life cycle without additional increase of cell titre that would lead to plant death. However, such kind of equilibrium is often violated during the infection. Undoubtedly, the identification of the players that disturb "homeostasis" between plants and pathogens within pathosystems is of particular importance for fundamental and agricultural sciences. One of such players in plant-*Pba* pathosystem appeared to be Cfa that specifically alters the plant physiology promoting excrescent proliferation of bacteria.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Human participants and animals studies Research did not involve Human Participants and/or Animals.

Informed consent The informed consent was not necessary.

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