Characterization of the infection cycle of *Phytophthora betacei* during disease development on tree tomato (*Solanum betaceum*).

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Phytophthora betacei is a recently described oomycete plant pathogen closely related to Phytophthora infestans sensu stricto. This plant pathogen infects tree tomato (Solanum betaceum) crops, but is not able to infect potatoes or tomatoes, the main hosts of P. infestans. The aim of this study was to characterize the infection cycle of P. betacei using microscopy and molecular approaches. To this end, tree tomato plants belonging to the susceptible ‘Comun’ accession were inoculated with a sporangial solution from several strains, and the progression of the disease was monitored based on six epidemiological parameters. The results indicated that strains from P. betacei display a highly variable disease phenotype. To understand the infection cycle at the cell and molecular level, one highly aggressive strain was selected based on the linear combination of the epidemiological parameters, and inoculation assays were performed. Samples collected from the inoculated plants 3, 6, 9, 12, 24, 48, 72, and 96 hpi were visualized using Scanning Electron Microscopy (SEM) and light Microscopy (trypan blue staining). Timing and progression of the disease was further validated with qRT-PCR by assessing the expression levels of infection-stage specific markers such as haustorium-specific membrane protein hmp1 and the cell cycle regulator Cdc14 along the infection cycle. Results indicated that the infection cycle of P. betacei differs from the closely related P. infestans in that the infection cycle of the former is characterized by a longer biotrophic stage, greater lesions and greater sporulation capacity compared to P. infestans when evaluated on the same host. Varying levels of expression were detected for both genes along the infection cycle, and their profile was consistent with the results observed on the whole plant inoculations. This study provides novel insights into the interaction between P. betacei and S. betaceum.
**Key words** Infection cycle, Phytophthora betacei, transcriptomics, tree tomato.
INTRODUCTION

During the last years, remarkable efforts have been made to understand the mechanisms underlying host colonization and pathogenesis during plant disease. In terms of host colonization, only the pathogens that are able to establish and evade recognition, or suppress host defense mechanisms are successful (Staskawicz, 2001). To accomplish this, plant pathogens use diverse strategies. For example, bacteria can only gain access to the plant through natural openings (stomata, hydathodes) or through wounds. Then, they establish themselves in the apoplast. In contrast, fungi and oomycetes can actively penetrate the plant epidermal cells and extend hyphae to eventually invaginate feeding structures (haustoria) in the host cell plasma membrane (Jones & Dangl, 2006). During these colonization and establishment processes, intimate molecular communication between the host and the pathogen occurs, leading to either plant resistance or disease. The latter is possible due to a diverse arsenal of specialized secreted pathogen molecules (effector proteins) able to counteract host defense mechanisms by altering the cell function or structure (Hogenhout et al., 2009).

Within the oomycetes, important research has been conducted concerning host colonization and pathogenicity (Hardham, 2001; Kamoun, 2007); especially for species within the Phytophthora genus (cf. Birch et al., 2009; Bozkurt et al., 2012; Fawke et al., 2015; Kamoun, 2007; Morgan & Kamoun, 2007). This genus is closely related to photosynthetic algae, is considered one of the most devastating plant pathogens (Kamoun, 2006), and is comprised of more than 100 species that have received special attention due to their economic and ecological impact (Martin et al., 2012). Some of the most representative species—and the diseases they cause—include: P. infestans (potato and tomato late blight),
*P. capsici* (blight and fruit rot of pepper), *P. sojae* (stem and root rot of soybean) (Lamour et al., 2007), *P. ramorum* (sudden oak death) (Grünwald et al., 2012), and *P. palmivora* (root rot of several plants, including oil palm) (Evangelisti et al., 2017).

Studies of disease establishment by *Phytophthora* pathogens made 30–40 years ago mapped out the major events that occur during the infection of host plants (Hardham, 2001), indicating that all *Phytophthora* species display a hemibiotrophic infection (Agrios, 2004; Fawke et al., 2015) where the pathogens feature biotrophy early in infection and necrotrophy in the later stages of host tissue colonization. More recent investigations have begun to expand our understanding of the typical hemibiotrophic infection stages for the economically relevant *Phytophthora* species by using cell and molecular biology approaches (cf. Avrova et al., 2003; Avrova et al., 2008; Chen et al., 2014; Chen et al., 2013; Hardham, 2001; Hayden et al., 2014; Judelson et al., 2008; Jupe et al., 2013; Kunjeti et al., 2012; Le Fevre et al., 2016; Xu et al., 2011; Ye et al., 2011). At the cellular and cytological level, these studies reveal that there are important differences among *Phytophthora* species regarding the timing of the hemibiotrophic cycle stages and the formation of the associated cell structures. For example, *P. sojae* shows to produce well developed haustoria 6hpi and fully invades susceptible soybean cultivars 24 hpi with the production of sexual structures (oospores) within the host tissue near by the initial infection site (Ye et al., 2011). In contrast, *P. infestans* develops mature haustoria 12hpi and invades a susceptible potato host 72 hpi, when the production of new asexual structures (sporangia) is evident (Grenville-Briggs & van West, 2005). At the molecular level, the main findings in these studies suggest tightly regulated transcriptional changes associated with molecular markers and pathogenicity and virulence factors. For example, the development of haustoria has been associated with upregulation of the
haustorial membrane protein gene (Hmp1) in species such as *P. infestans, P. palmivora* and *P. capsici*. Likewise, the cell cycle regulator gene (*Cdc14*), has also been found to be upregulated, except later in the infection cycle during sporangia production for all the mentioned species (Ah Fong & Judelson, 2003; Jupe et al., 2013; Le Fevre et al., 2016). Lastly, large-scale expression data provided these studies with evidence of transcriptional regulation of effector-coding genes during the infection of *Phytophthora* species on their natural hosts, supporting the hypothesis that the infection features stage-specific transcriptional programs (Randall et al., 2005). The accessibility to oomycete pathogen genome sequences, combined with gene expression data from both pathogen and host throughout the course of infection, can serve as a basis for identification and curation of effector genes that may have important roles in both virulence and avirulence.

Recently, phylogenetic, population genetics and morphological approaches allowed the description of a new species of *Phytophthora* (*P. betacei*) within the clade 1c (Mideros et al, 2017). This species coexists with *P. infestans* in South America, and has been associated with late blight on tree tomato (*Solanum betaceum*), a semi-domesticated South American fruit crop. Interestingly, *P. betacei* has not been reported infecting potatoes or tomatoes (the main hosts of *P. infestans*, its sister species), suggesting that this species originated through ecological speciation by host specialization, which appear to be the main mechanisms maintaining the divergence between these two species (Mideros et al, 2017; Restrepo et al., 2014). However, the overall colonization mechanisms underlying the pathosystem *P. betacei* – *S. betaceum* remain unexplored, and there is no information available on the transcriptional changes that allow such specialized interaction.
Here, we characterized the infection cycle and assessed the expression profile of secreted putative proteins of the recently described oomycete plant pathogen *P. betacei* while infecting its natural host, tree tomato. First, we measured six epidemiological parameters on inoculated susceptible tree tomato plants to describe the infection cycle and progression of the disease. Second, we closely followed the infection at different stages of pathogenesis (pre-penetration, penetration, and colonization) by visualizing inoculated plant material with light and scanning electron microscope (SEM), and further validated the results by quantifying the expression levels of two stage-specific molecular markers. Lastly, we tested the hypothesis that this pathogen bears a different set of effectors compared to those found in *P. infestans*. To demonstrate this, we reported and evaluated a profile of secreted putative effector proteins that showed to be up and down regulated along the infection cycle according to RNA-seq data.

MATERIALS AND METHODS

Strains selection and maintenance

Eleven strains were selected from the *Phytophthora* collection maintained in the Universidad de Los Andes museum. All *P. betacei* strains used in this study (Supplementary Table 1) were routinely cultured at 18 °C in tree tomato agar medium (1.8% bacteriological agar, 1.8% sucrose, 0.05% CaCO$_3$, 20% tree tomato juice). Three *P. infestans* strains isolated from potato fields (Supplementary Table 1) were included to compare the disease progression, and were maintained in potato dextrose agar (PDA) (Oxoid, Waltham, MA, USA) and clarified V8 agar (2% bacteriological agar, 10% clarified V8, 0.1% CaCO$_3$). To
actively maintain the virulence condition of the strains, *P. betacei* was kept on tree tomato leaves from the ‘Comun’ accession (known to be susceptible) and *P. infestans* was kept on potato leaves prior to the inoculation assays. Strains were routinely transferred to fresh leaves by inoculating four 20 μL droplets of a non-adjusted sporangial suspension on the abaxial side of a tree tomato or potato leaf, these were then placed inside a petri dish, which created a moist chamber, and were incubated at 18°C until they showed sporulation signs that were used to prepare adjusted sporangial suspensions.

### Growth conditions of plant material

Susceptible tree tomato (*Solanum betaceum*) plants, belonging ‘Comun’ accession, were used for all experiments. Tree tomato plants were obtained from certified seeds. All seeds were submerged in distilled water for 24 hours before germination, as suggested by the manufacturer (Impulsemillas, Bogotá, Colombia). Subsequently, seeds were placed in an enriched substrate (peat) to induce germination and were then transplanted to individual pots. Germination and growth were carried out under greenhouse conditions (12h light period, 18°C). All experiments in this study were performed on eight to ten-week old tree tomato plants.

### Disease progression of *P. betacei* strains on tree tomato

#### Plant Inoculation trials

A complete randomized design was used to test the variation of disease progression of *P. betacei* strains (Table S1). For all experiments, a sporangial suspension of each *P. betacei* strain consisting of $3.5 \times 10^5$ sporangia mL$^{-1}$ (Mideros et al., 2017) was prepared
using a hemocytometer. Due to poor sporulation, *P. infestans* sporangial suspensions were
adjusted to $2.5 \times 10^5$ sporangia mL$^{-1}$ and incubated 3 h at 4°C to promote zoospore release.
After incubation, each suspension was inoculated on three plants as follows: three leaves of
the same plant were drop-inoculated on the abaxial side using four 20 µL droplets of the
adjusted suspension. A total of nine leaves were inoculated per sporangial suspension.
Subsequently, Inoculated plants were placed inside a growth chamber (Percival, Perry, Iowa,
USA) at 17 ± 2 °C, 80% relative humidity, and 12 h light period. Plants were individually
covered with a plastic sheet throughout the entire experiment to maintain the appropriate
conditions and to avoid cross contamination. The experiment was performed separately three
times to ensure the reproducibility of the data for a total of nine inoculated plants per strain.

For each strain, disease progression was documented for nine days based on the total
lesion area (LA, mm$^2$ at day nine), lesion growth rate (LGR, mm$^2$ day$^{-1}$), incubation period
(IP, time between inoculation and first appearance of symptoms), sporulation capacity (SC, sporangia mm$^{-2}$), latency period (LP, time between inoculation and first sporulation), and
sporulation rate (SR, sporangia mm$^{-2}$ day$^{-1}$) (Mideros et al., 2017). Total lesion area (LA)
was determined using the ImageJ 1.49 software (Research Services Branch, National
Institute of Mental Health, Bethesda, Maryland, USA) by measuring the necrotic areas, and
sporulation capacity (SC) by rinsing sporangia from sporulating lesions on 1 mL of distilled
water, counting them using a hemocytometer, and then dividing them over the lesion area.
LGR was estimated by regressing LA for each lesion on each plant over time. The slope of
the regression line was used as a proxy for the LGR.

Statistical analyses
Summary statistics were calculated for each measured variable per strain and per species. Subsequently, the normality of the residuals was assessed for each epidemiological parameter using the function “shapiro.test” available in the package stats from R software (R core team, 2015). This test indicated that these were not normally distributed \((p < 0.01)\), and thus to identify differences among the strains and between the species a Kruskal-Wallis test was performed. The Nemeyi post hoc test from the Pairwise Multiple Comparison of Mean Ranks Package (‘pmcmr’) implemented in R (Pohlert, 2014) was used to assess particular differences among strains.

Next, we established whether the strains from \(P.\ betacei\) and \(P.\ infestans\) clustered together according to a combination of the epidemiological components. This was achieved by visualizing all the epidemiological parameters in a bidimensional plane. To this end, the correlation among epidemiological parameters was estimated using Spearman’s correlation coefficients, and a Linear Discriminant Analysis (LDA) was implemented using a matrix of six components and a total of 14 individuals (eleven strains for \(P.\ betacei\), and three strains for \(P.\ infestans\)). Analyses were conducted using the “lda” function from the package ‘mass’ in R (Venables & Ripley, 2002).

Lastly, to correctly identify groups of similar strains, a K-means analysis was implemented over the values obtained from the LDA (MacKay, 2003). First, the number of ideal clusters \(K\) for the data was selected by implementing the elbow method, that relies on the explained variance. Here, the variance was measured as sum of squares within a cluster and the maximum number of clusters allowed was 14, assuming each strain would group into a unique cluster. Once the \(K\) value was selected, the “kmeans” function from the package ‘stats’ in R (parameters set.seed = 30, \(K=5\), nstart = 50) was used to assign data points to
clusters. From here, one *P. betacei* and one *P. infestans* strains belonging to highly different clusters were selected to be further studied.

**Microscopic characterization of *P. betacei* infection cycle**

*Light and scanning electron microscopy*

To investigate the infection cycle of the selected *P. betacei* and *P. infestans* strains at the cellular level, we assessed zoospore establishment and encystment, spore germination and host penetration, plant colonization and nutrient acquisition (formation of haustoria), and sporulation using light and electron microscopy. For this purpose, each selected strain was inoculated as described above (see disease progression section) and two sets of leaf discs were excised after 3, 6, 9, 12, 24, 48, 72, and 96 hours post inoculation (hpi).

For scanning electron microscopy, discs from one of the sets were fixated with 2.5% glutaraldehyde in 0.1 M Sorenson’s buffer (pH= 7.2) for 24 h at room temperature. Subsequently, they were washed twice, each time for 10 minutes, in the same buffer. After fixation, the samples were dehydrated in a graded ethanol series as follows: 50%, 70%, 90%, and 95% for a period of 30 min in each series, and two changes of 2 hours each in a 100% series. The samples were critical point dried using CO2. The fixed material was mounted on stubs using double-sided carbon tape and coated with gold/palladium in a sputter coater system in a high-vacuum chamber for 150 s at 9 mA (Soylu, Soylu, & Kurt, 2006). The samples were then visualized using a Scanning Electron Microscope (SEM) accelerating voltage 20kv (JEOL Microscope, model JSM 6490-LV, Peabod, MA, USA).

For light microscopy, discs from the remaining set were subjected to trypan blue staining. The discs were fixed in acetic acid (99%)/ethanol (96%) 3:1) until they had been
fully decolorized (approximately 24h, the decolorizing solution was changed every 12h),
followed by dehydration in an ethanol series (50% and 70%) for a period of 24h each.
Staining was then performed using 0.04% trypan blue in lactophenol/ethanol (96%) (1:2)
solution for 2 minutes. The leaf pieces were vacuum infiltrated at room temperature for 5
min and incubated in the trypan blue solution for another 2h at 4°C. Leaf pieces were
mounted in glycerol 50% (Colon et al., 1992) and visualized using a light microscope (ZEISS
Axioskop 40, Goettingen, Germany).

Quantitative RT-PCR during the infection cycle
The infection cycle was further studied by assessing the expression levels of two
previously reported infection-stage specific markers using qRT-PCR. The markers studied
were the haustorium-specific membrane protein Pbhmmp1 (Avrova et al., 2008) and the cell
cycle regulator Pbcycl14 (Ah Fong & Judelson, 2003). These marker genes were first
searched on the P. betacei genome using Basic Local Alignment Search Tool BLAST
(Altschul, Gish, Miller, Myers, & Lipman, 1990) using the homologues found in P. infestans
as a query. Subsequently, to assess their expression levels, pieces of inoculated tissue were
harvested from plants 6, 12, 18, 24, 48, and 96 hpi, and placed in liquid nitrogen immediately.
The RNase Plant MiniKit® (Qiagen, Germantown, MD, USA) was used to extract total
RNA from infected tissue and mycelia according to the manufacturer’s instructions. The
integrity and yield of the RNA was assessed using a bioanalyzer. Extracted samples were
treated with DNase I prior to cDNA synthesis. cDNA was generated using the High-Capacity
cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) with 1 µg of
DNase-treated RNA. A standard qRT-PCR was performed to examine the gene expression
levels of *Pbhmpl* and *Pbcdc14* by using Maxima SYBR Green qPCR (Thermo Scientific, Waltham, MA, USA) and a 7500fast thermocycler (Applied Biosystems, Foster City, CA, USA). The Actin gene *PbactA* was used as an endogenous control gene, as described previously (Bos et al., 2010; Gilroy et al., 2011). The primer combinations and genes are listed in supplementary Table 2. Reactions consisted of a final volume of 15 µL including 2 µL cDNA (500 ng total), 0.45 µL of each 0.3 µM primer, 5 µL of Maxima SYBR green qPCR master mix without ROX, 0.03 µL of 10 nM ROX, and nuclease-free water to complete the volume. The PCR parameters were as follows: 95 °C, 15 min; 95 °C, 15 s; 60 °C, 30 s; 72 °C, 30 s (40 cycles). Melting curve analyses were performed on every run to confirm a single product (60–95°C) reading every 1°C. Gene expression Ct values were normalized with actin and made relative to mycelia (Bos et al., 2010). Quantification analyses were performed according to the Pfaffl method (Pfaffl, 2001) and using the software REST (Pfaffl et al., 2002).

**Expression profile of putative effector proteins**

*Library preparation and RNA sequencing*

To identify transcripts along the infection cycle, a RNAseq analysis was performed on leaf tissue harvested from inoculated plants at 6, 12, 18, 24, 72 and 96 hpi, as well as from uninoculated plant material, mycelia and sporangia. For the RNA extractions, three pieces of inoculated tissue were harvested from each plant (one piece per inoculated leave, nine pieces total) at each selected time point and placed in liquid nitrogen immediately. Total RNA was obtained from each sample using the RNeasy Plant Mini Kit® (Qiagen, Germantown, MD,
USA) according to the manufacturer’s instructions. The purified RNA was treated with DNase I, and its integrity and yield were measured using a 2100 bioanalyzer (Agilent, Waldbronn, Germany). *S. betaceum* and *P. betacei* messenger RNAs (mRNAs) were purified using Poly(A) selection from the total RNA sample and then fragmented. cDNA library preparation was performed with the non-directional NEBNext® Ultra™ RNA Library Prep Kit for Illumina® (New England Biolabs, Hitchin, UK) according to the manufacturer’s protocol and sequenced on an Illumina HiSeq4000 system (Illumina, San Diego, CA) over a paired-end library (insert size of ~250 -300 bp) for three biological replicates. A total of 30M reads were obtained per plant sample, and between 60-90M reads for the mycelia, spore and inoculated tissues.

**De novo transcriptome assembly**

To define the quality parameters of the reads to be used in the transcriptome assembly, all reads (~1342 M) were quality-assessed using the FASTQC tool v.0.11.2 (Babraham Bioinformatics, Cambridge, UK). Potential contaminants were then identified by screening the reads against the SILVA data bases using ‘bbduk.sh’ from the BBtools package (kmer = 27) (Joint Genome Institute). The sequences of the identified contaminants were obtained from the RefSeq data base, and used to further clean the reads using the same tool. Lastly, a step of trimming and filtering was performed using Trimmomatic v.0.36 (Bolger et al., 2017) with the following parameters: threads 5, phred33, HEADCROP:10, MINLEN:80. The remaining (~1310 M) reads were subjected to FASQC and used in downstream analyses.
Clean reads from the mycelia, spore, inoculated plant and leaf samples were used in a de-novo assembly of reference transcriptomes for *P. betacei* and *S. betaceum*, respectively using the Trinity package (Grabherr et al., 2013; Haas et al., 2013) (v2.4) with kmer=25. The *S. betaceum* transcriptome assembly was then used to exclude plant reads from the inoculated samples library by implementing the pseudoalignment function ‘pseudo’ from Kallisto (Bray et al., 2016). Since plant transcripts were still present, a mapping round of HISAT2 (Kim et al., 2015) of the unmapped reads was performed against the *Solanum tuberosum* (v4.03), *S. lycopersicum* (v3.0) and *C. annuum* (v2.0) reference genomes. The filtered reads from inoculated plants, mycelia and spore (~404 M) were used in a de-novo assembly with Trinity (v2.4) on a high-RAM server with kmer length=25. In all cases, in silico read normalization was used during transcriptome assembly due to large number of input reads. The resulting assembly was additionally checked for plant contamination using blastn search against the plant division of the National Center for Biotechnology Information (NCBI) RefSeq genomic database (Evangelisti et al., 2017). Trinity genes having significant similarity (e-value threshold ≤10^-5) to plant sequences were removed from the transcriptome.

**De novo assembly statistics and transcriptome completeness**

General statistics of the assembly were obtained using the “TrinityStats.pl” script provided with Trinity and independently using the software Transrate (Smith-unna et al., 2016). Furthermore, the number of full length transcripts was estimated by examining the extent of top-matching blastx alignments against the SwissProt data base, the percent of the target being aligned to the best matching Trinity transcript, and by grouping blast hits to improve sequence coverage (Haas et al., 2013). These scripts are available with the Trinity utilities.
RESULTS

*Phytophthora betacei* displays a highly variable phenotype while colonizing a susceptible host

With the aim to understand the infection cycle of *P. betacei*, we screened a collection of strains based on six epidemiological parameters (LA, LGR, IP, SC, SR, LP). Analysis of the normality for each variable showed that the residuals were not normally distributed (Shapiro Wilk test, p-value < 0.01) and thus non-parametric statistical analyses were implemented. All p-values were corrected for multiple comparisons using the Bonferroni correction method. All the analyses were performed on pooled data from three replicates.

The median and interquartile range of all six epidemiological parameters are presented for each evaluated strain on table 1, and for each species on table 2.

Furthermore, we evaluated whether there were significant differences among strains for each epidemiological parameter. Our results indicated that most strains from *P. betacei* displayed differences regarding LA (Kruskal-Wallis $\chi^2 = 50.611$, df = 13, p < 2.3 x 10^{-6}), IP (Kruskal-Wallis $\chi^2 = 73.425$, df = 13, p < 1.86 x 10^{-10}), and SC (Kruskal-Wallis $\chi^2 = 39.072$, df = 13, p < 1.94 x 10^{-4}) when compared to each other and when compared to strains from *P. infestans*. In contrast, no differences were detected among *P. infestans* strains. As a species, *P. betacei* differed from *P. infestans* for LA (Kruskal-Wallis $\chi^2 = 16.584$, df = 1, p < 4.655 x 10^{-5}), IP (Kruskal-Wallis $\chi^2 = 23.076$, df = 12, p < 1.577 x 10^{-6}), SC (Kruskal-Wallis $\chi^2 = 97.875$, df = 12, p < 2.2 x 10^{-16}), and SC (Kruskal-Wallis $\chi^2 = 15.642$, df = 1, p < 7.65 x 10^{-5}).
Spearman’s correlation values of the epidemiological parameters, while significant in most cases ($p < 0.01$), did not reveal strong correlations between the variables ($\rho < 0.5$). Only LGR showed to be strongly correlated with LA and was excluded from the linear discriminant analysis. The linear combination of the remaining variables indicated that $79.32\%$ of variance among strains was explained by two discriminants. Figure 1A shows the plot of the first and the second discriminant components for $P. \text{betacei}$ and $P. \text{infestans}$ strains. Clearly, $P. \text{betacei}$ has more variable infection pattern as the strains do not cluster together (circles), compared to that in $P. \text{infestans}$, where all the strains grouped together (triangles). The first linear discriminant function (LD1, 68.39\%) characterized the strains based on IP. For the second linear discriminant function (LD2, 10.93\%), LA and IP where the variables best discriminating among strains.

The grouping results were supported by a K-means clustering analysis, where five clusters were identified within the data (Figure 1B). As expected, strains from $P. \text{infestans}$ clustered together or in neighboring groups, while $P. \text{betacei}$ showed to be more dispersed, and in some cases (strains P8077, P9127, P9153 and P9151) were grouped with $P. \text{infestans}$. The first and second clusters included all the $P. \text{betacei}$ strains with intermediate phenotypes for both epidemiological parameters and all $P. \text{infestans}$, the third included strains with larger lesion sizes and long incubation periods, the fifth included strains with small lesion sizes and long incubation periods. The fourth cluster was found to be outlier data points.

Distribution of the values for the LA and IP are shown in figure 2. Overall, $P. \text{betacei}$ clearly shows a higher intra-species phenotypic differentiation for LA given that the values range from small to large, but not in IP where most strains take longer to produce any
symptoms. In contrast, strains from *P. infestans* performed equally when compared to each other regarding LA, and minor differences for IP among strains were found to be non-significant (Nemeyi Test p > 0.01).

From these combined results, we selected strain N99035 from *P. betacei* (third cluster) and Z32 from *P. infestans* (second cluster) to be further studied using microscopic and molecular approaches.

*Phytophthora betacei* and *Phytophthora infestans* – tree tomato interactions feature a hemibiotrophic infection cycle

The interaction between *P. betacei* strain N9035 and *P. infestans* Z-32 with tree tomato (*Solanum betaceum*) was investigated in time-course experiments. Inoculations followed by phenotypic analyses across time points suggested that the evaluated strains display a hemibiotrophic infection cycle. Particularly, N9035 and Z-32 presented differences in the timing of biotrophic and necrotrophic stages. In the early stages of infection (up to 72 hpi), *P. betacei* featured a biotrophic phase during which host tissue appeared healthy and unaffected, followed by a necrotrophic phase (>96 hpi) where water soaking, necrosis and tissue collapse were evident. New sporangia were visible on the rim of the necrotic area at 120 hpi (Figure 3A-E). Meanwhile, *P. infestans* rapidly switched from biotrophy to necrotrophy (>24 hpi), and the tissue rapidly decayed; however, sporangia were not detected with a naked eye (Figure 4A-D).

Based on these observations, the ability of N9035 and Z-32 to form penetration, feeding, colonization, and dispersal/reproduction structures *in planta* was examined by
visualizing infected plant material using light and Scanning Electron Microscopy. The findings indicated that both strains were capable to form appresoria, haustoria, and invasive hyphae. Cysts were observed as early as 3hpi, germinated cysts 6hpi, and appresoria and haustoria >9hpi (Figures 3F-L and 4E-H). Microscopic inspection of leaf tissue in the later infection stages revealed significant colonization with formation of sporangia 120 and 72 hpi for N9035 and Z-32, respectively (Figures 3E and 4I). From this, we concluded that P. betacei exerted a hemibiotrophic infection cycle in tree tomato. We found both direct and indirect germination of the sporangia that led to the formation of appresoria and haustoria on asymptomatic tissue, suggesting that all strains established a biotrophic interaction with the host. Later, we found tissue collapse and necrosis accompanied with sporulation in N9035 and Z-32 as sign of a necrotrophic phase and the end of the infection cycle. An interesting observation was made on the timing of zoospore release. P. betacei did not appear to release the zoospores from the sporangium until after 3 hours of reaching the host, while P. infestans appeared to liberate the zoospores while on the sporangial solution previous to the inoculation (Figures 3F and 4E).

The infection stages of P. betacei and P. infestans on tree tomato are marked by expression changes in related molecular markers

With the aim to support the epidemiological parameters assessment and microscopic studies, the expression of the P. infestans orthologues of the haustorial membrane protein 1 (Hmp1) and the cell cycle regulator phosphatase (Cdc14) was quantified. The qRT-PCR results indicated that the transcript encoding Hmp1 peaked at 12 hpi with a fold-change ~ 4 for N9035, and ~ 2.5 for Z-32. These findings correlated well with the observation of the first haustoria on the plant’s tissue. After this time, the gene became down-regulated in N9035
(Figure 5A), when the inoculated material started to become necrotic. In the case of Z-32, the expression pattern of *Hmp1* was not consistent with the expected as it peaked again 72hpi. By contrast, *Cdc14* was highly induced later during the infection cycle. The first detection was done at 12 hpi with a fold change ~5 for N9035, when some sporangia were still be present from the first inoculation, and then later at 96 (fold change ~15) and 72 (fold change ~12) hpi, for N9035 and Z-32 respectively (Figure 5B). Again, these results correlated with the observation of sporangia on the necrotic tissue at the given time points. Taken together, these results further supported the conclusion that *P. betacei* displayed a hemibiotrophic lifestyle on *S. betaceum* plants.

*De novo* assembly of *Phytophthora betacei* and *Solanum betaceum* transcriptomes

To understand the processes underpinning *P. betacei* infection and disease progression on tree tomato, a RNA-seq experiment was designed and gene expression changes in both organisms were measured. After cleaning and trimming, 2.45% of the reads were identified as contaminants (mainly rRNA). Furthermore, the assembly of the *S. betaceum* transcriptome (~114 M reads) yielded 241,871 transcripts belonging to 113,756 Trinity genes. A total of 8,793 full-length transcripts were identified according to the SwissProt data base, where 673 were covered by 90% of their length. On the other hand, *P. betacei* produced an ‘anoxic culture’ and a ‘full complexity’ transcriptomes. The former produced 135,443 Trinity transcripts and 50,079 Trinity genes, of which 1559 were covered by 90% of their length. The latter yielded 957,053 transcripts and 399,356 Trinity genes with 3983 being covered by >90% of their length. Summary assembly statistics of the three reference transcriptomes are presented in table 3.
DISCUSSION

In this study, an integrative approach that leveraged microscopy and molecular assays was employed to successfully describe the infection cycle of *P. betacei* on tree tomato. The data collected highlighted strong variation of the phenotypes across strains from *P. betacei* when infecting a susceptible host, and revealed a more stable phenotype distribution among strains from *P. infestans*. Furthermore, a detailed investigation of the infection cycle of *P. betacei* and *P. infestans* allowed us to clearly identify the biotrophic and necrotrophic stages by visualizing infected material and by assessing the expression level of two marker genes. At this point, we concluded that *P. betacei* exerts a typical hemibiotrophic infection with a long biotrophic period characterized by the presence of feeding structures, followed by the necrotrophic stage where the pathogen sporulates to start a new cycle.

*Phytophthora betacei* has been described as a specialist of tree tomato, a semi-domesticated crop endemic to the northern Andes (Mideros et al., 2017). All *P. betacei* isolates tested in this study infected and caused disease on a susceptible tree tomato host; however, large variation in the phenotype was observed for all the measured epidemiological components. These results are not striking given that previous studies have reported the possibility of variability in aggressiveness among isolates belonging to the same species or sharing similar genotypes (Pariaud et al., 2009). For example (Carlisle, Cooke, Watson, & Brown, 2002) demonstrated significant variation for foliar aggressiveness (measured as lesion expansion rate, latent period, sporulation and infection efficiency) exists within the Northern Ireland population of *P. infestans* when inoculated on the same potato cultivar. The variation was explained in terms of geographic origin of the strain. Similar findings were reported for the leaf rust pathogen *Puccinia striiformis f. sp. tritici* (Milus, Seyran, & Mcnew,
2006) where isolates belonging to the same pathotype exhibited different latency periods. This strong reliance on diversity may be interpreted as the result of balancing selection for epidemiological components involved in trade-offs acting at different stages of the life cycle, and hence as a way of partitioning the risk of extinction in unstable environments (Andrivon et al., 2013; Chakraborty, 2013), such as host undergoing domestication. Additionally, we can attribute this variability to the performance of experiments on complete tree tomato plants, and to its defense mechanisms since differences can be less distinguishable on a less resistant cultivar (Pariaud et al., 2009). The tree tomato accession used in this study has not been accurately phenotyped or genotyped, and thus we relied on undocumented susceptibility information that can bias our results.

A key aspect of the development of sustainable control measures for Phytophthora diseases is the comprehensive understanding of the infection process at a cellular and molecular level (Hardham, 2001). *P. betacei* follows a typical hemibiotrophic infection cycle where it first contacts the plant and becomes firmly attached to the surface, then penetrates and colonizes the plant, while up taking the nutrients it needs for growth and sporulation, to later produce spores that can initialize a new cycle. Using light and scanning electron microscopy coupled with gene expression analyses, we found evidence of a distinct biotrophic phase marked by the appearance of haustoria at 9hpi, to later transition to a necrotrophic phase starting a 72 hpi. These biotrophic phase is comparable with observations made in other *Phytophthora* species such as *P. infestans*, *P. capsici*, *P. palmivora*, and *P. sojae* (Ali et al., 2017; Evangelisti et al., 2017; Jupe et al., 2013; Kunjeti et al., 2012; Ye et al., 2011; Zuluaga et al., 2016). However, it was noted that *P. betacei* establishes a longer biotrophic phase, and therefore has a longer infection cycle compared to the other species...
when infecting their natural hosts, and when compared to the cycle of *P. infestans* on tree
tomato (where the biotrophy occurs only for 24 hours and the cycle ends with the appearance
of new sporangia 72hpi). This extended level of biotrophy could be an indicator for the level
of relative adaptation among isolates on the host (Kröner, Mabon, Corbiere, Montarry, &
Andrivon, 2017), and could suggest the presence of specific adaptations required by
*P. betacei* to thrive on its host.

The use of molecular markers contributed to the better understanding of the colonization
process and validated the phenotypic observations. The presence of biotrophic feeding
structures (haustoria) was accompanied with the upregulation of the gene encoding the
haustorium membrane protein (*Hmp1*). It has been proposed that this protein is involved in
the stability of the plasma membrane of the pathogen and that it localizes only in regions
where infection vesicles and haustoria are observed (Avrova et al., 2008). These
observations support the hypothesis that a compatible plant-pathogen interaction occurs
between *P. betacei* and *P. infestans* with tree tomato, and that *P. betacei* might be producing
a larger number of haustoria on the plant than *P. infestans*, given that the expression fold
change of the later was significantly smaller. Usually, the degree of compatibility of the
interaction influences the number of haustoria that are formed (Hardham, 2001).

Comparably, the expression profile of the cell cycle phosphatase 14 (*Cdc14*), accurately
supported the results for sporulation by showing to be upregulated 96 hpi for *P. betacei* and
72hpi for *P. infestans*. This protein plays an essential role during asexual sporulation and
The formation of sporangia is an essential developmental process required for propagation
during a *Phytophthora* infection. The sporulation marker gene *cdc14* was significantly more
expressed in *P. betacei* than in *P. infestans*, supporting the conclusion that *P. betacei* successfully draws nutrients from the plant allowing the production of new sporangia that can start a new cycle. The completion of the infection cycle with abundant production of new spores can be an indicator that this isolate of *P. betacei* does not face a trade-off scenario between virulence and aggressiveness when infecting a susceptible host as it remains both aggressive (large lesions, abundant feeding structures) and virulent (abundant sporangia). In the case of *P. infestans*, producing a large number of spores might imply a cost, and therefore it could be adapting its infection cycle on tree tomato producing a lower amount of sporangia but more rapidly (Andrivon et al., 2013).

This study highlights the importance to use the combined effect of several epidemiological parameters in the characterization and selection of *Phytophthora betacei* strains, as greater differences can be detected when the contribution of each variable is leveraged (Miller, Johnson, & Hamm, 1998). Natural variation in the infection phenotype has been observed within the *Phytophthora* genus, and might be an indicator of the level of adaptation of the pathogen. Furthermore, a clear hemibiotrophic infection cycle was confirmed by the presence of haustoria during an asymptomatic period, followed by necrosis and sporulation. The mechanisms behind the extended biotrophic stage remain unexplored and require special attention as they might be involved in the host specificity of *P. betacei*. Particularly, the early stages of host recognition that seem to play an important role during zoospore release, as these were only liberated from the sporangia 3hpi and could not be produced *in vitro*. Lastly, the use of genetic markers provided strong support to the phenotypic results, and demonstrated that the infection cycle of *P. betacei* is a tightly
regulated process. RNA-seq experiments will provide detailed information of the transcriptional changes *P. betacei* undergoes while colonizing its natural host.

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Pfaffl, M. W., Horgan, G. W., & Dempfle, L. (2002). Relative expression software tool (REST©) for group-wise comparison and statistical analysis of relative expression results in real-time PCR, 30(9).


http://doi.org/10.1198/tech.2003.s33


http://doi.org/10.1038/nature10158


Table 1. Median value of each evaluated epidemiological parameter among strains. Eleven *P. betacei*, and three *P. infestans* strains inoculated on susceptible tree tomato (Amarillo accession) plants. The first and third quartile are indicated in the parenthesis as a dispersion measurement. The size of the sample (n) might differ among strains due to lack of symptoms and signs in some of the plants.

<table>
<thead>
<tr>
<th>Epidemiological parameter</th>
<th>Species</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>P. betacei</em></td>
<td><em>P. infestans</em></td>
</tr>
<tr>
<td></td>
<td>P8093</td>
<td>P9127</td>
</tr>
<tr>
<td>LA (mm²)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>n=1</td>
<td>n=9</td>
</tr>
<tr>
<td></td>
<td>24.7</td>
<td>33.64</td>
</tr>
<tr>
<td>LGR (mm²h⁻¹)</td>
<td>0.11</td>
<td>0.15</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(0.07-)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(0.19-)</td>
</tr>
<tr>
<td>IP (hpi)</td>
<td>48</td>
<td>96.00</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(53.3-)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(120-)</td>
</tr>
<tr>
<td>SC (sp mm⁻²)</td>
<td>0</td>
<td>509.69</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(405.1-)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(815.7-)</td>
</tr>
<tr>
<td>SR (sp hpi⁻¹)</td>
<td>0</td>
<td>39.78</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(34.72-)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(119.6-)</td>
</tr>
<tr>
<td>LP (hpi)</td>
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<td>192</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(162-)</td>
</tr>
</tbody>
</table>
Table 2. Median value of each evaluated epidemiological parameter between species.

The first and third quartile are indicated in the parenthesis as a dispersion measurement.

<table>
<thead>
<tr>
<th>Epidemiological parameter</th>
<th>Species</th>
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</thead>
<tbody>
<tr>
<td></td>
<td><em>P. betacei</em></td>
</tr>
<tr>
<td>Lesion Area (mm²)</td>
<td>n=11</td>
</tr>
<tr>
<td></td>
<td>18.8 (15.24-42.36)</td>
</tr>
<tr>
<td>Lesion Growth Rate (mm² h⁻¹)</td>
<td>0.087 (0.07-0.19)</td>
</tr>
<tr>
<td>Incubation Period (hpi)</td>
<td>81.19 (53.33-112.0)</td>
</tr>
<tr>
<td>Sporulation Capacity (sp mm⁻²)</td>
<td>405.1 (0-509.7)</td>
</tr>
<tr>
<td>Sporulation Rate (sp h⁻¹)</td>
<td>39.8 (0-100.6)</td>
</tr>
<tr>
<td>Latency Period (hpi)</td>
<td>185.1 (0-216)</td>
</tr>
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</table>
Table 3. De novo transcriptome assembly statistics for *P. betacei* and *S. betaceum*

<table>
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<th>Metric</th>
<th>Value</th>
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<td></td>
<td><em>P. betacei</em></td>
</tr>
<tr>
<td></td>
<td>Full</td>
</tr>
<tr>
<td>Total Trinity genes</td>
<td>399,536</td>
</tr>
<tr>
<td>Total Trinity transcripts</td>
<td>957,073</td>
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<tr>
<td>Percent GC</td>
<td>44.60 %</td>
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<tr>
<td>Smallest contig</td>
<td>201</td>
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<tr>
<td>Largest contig</td>
<td>29,427</td>
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<tr>
<td>Statistics based on all transcripts</td>
<td></td>
</tr>
<tr>
<td>Contig N50</td>
<td>678</td>
</tr>
<tr>
<td>Median contig size</td>
<td>370</td>
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<tr>
<td>Average contig size</td>
<td>563.36</td>
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<tr>
<td>Total assembled bases</td>
<td>539,176,830</td>
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<tr>
<td>Statistics based on the longest isoform per gene</td>
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<tr>
<td>Contig N50</td>
<td>566</td>
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<tr>
<td>Median contig size</td>
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<tr>
<td>Average contig size</td>
<td>493.16</td>
</tr>
<tr>
<td>Total assembled bases</td>
<td>197,036,414</td>
</tr>
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</table>
FIGURES LEGENDS

Figure 1. Clustering analysis of Phytophthora strains based on five epidemiological parameters. A) Linear Discriminant Analysis (LDA) showing the first and second linear discriminants. The Phytophthora strains were differentiated by Lesion Area (LA) and Sporulation Capacity (SC) on the first component (LD1=61.56%), and by Incubation Period (IP) on the second component (LD2=13.75%). Due to collinearity, Lesion Growth Rate (LGR) was excluded from the analysis. B) K-means Clustering. The linear discriminant values from each strain were used to assign 5 clusters. The ideal number of clusters was selected based on total within cluster sum of squares. Each cluster represents a group of similar strains.

Figure 2. Phytophthora betacei shows variability in lesion area, incubation period and sporulation capacity. A) Lesion Area (mm$^2$) and B) Incubation period (hours post inoculation). From these, strain N9035 from P. betacei and Z32 from P. infestans were selected to be further characterized.

Figure 3. Phytophthora betacei features a hemibiotrophic life style. A-E) Susceptible Tree tomato plants inoculated with sporangia suspension of P. betacei at 24, 48, 72, 96, 120 hours post inoculation (hpi). F-L) Detail of the infection process of P. betacei under light (F, G, J, K, L) and Scanning Electron Microscopy – SEM (H, I) at 0, 3, 6, 9, 12 hpi. Sp=Sporangia, St=Stomata, Cy=Cyst, Gc=Germinated Cyst, Ap=Appresorium, Ha=Haustoria, Gs=Germinated Sporangia.
Figure 4. *Phytophthora infestans* features a hemibiotrophic life style on a non-natural host. A-D) Susceptible Tree tomato plants inoculated with sporangia suspension of *P. infestans* at 24, 48, 72, 96 hours post inoculation (hpi). E-I) Detail of the infection process of *P. infestans* under light (E-F) and Scanning Electron Microscopy – SEM (I) at 0, 3, 6, 9, 72 hpi. Sp=Sporangia, St=Stomata, Cy=Cyst, Gc=Germinated Cyst, Ap=Appresorium, Ha=Haustoria, Gs= Germinated Sporangia, Esp = Emerging sporangia.

Figure 5. Validation of infection cycle stages by qRT-PCR. Expression relative to actin was assessed for the lifestyle marker genes Haustorium membrane protein *Hmp1* (A) and Cell cycle phosphatase *Cdc14* (B), on time-course experiments for *P. betacei* (orange) and *P. infestans* (gray). Quantitative RT-PCR experiments were performed in duplicates. Bars represent the Standard Error (SE).
Figure 1.
Figure 2.

A

Lesion Area

B

Incubation Period
Figure 3.
Figure 4.
Figure 5.
**SUPPLEMENTARY TABLES**

**Supplementary Table 1.** *Phytophthora betacei* and *Phytophthora infestans* strains used in this study.

<table>
<thead>
<tr>
<th>Strain ID</th>
<th>Host</th>
<th>Species</th>
<th>Location</th>
<th>Reference</th>
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<tbody>
<tr>
<td>P9127</td>
<td>Solanum betaceum</td>
<td>P.betacei</td>
<td>Putumayo, Colombia</td>
<td>(Mideros et al., 2016)</td>
</tr>
<tr>
<td>P8084</td>
<td>Solanum betaceum</td>
<td>P.betacei</td>
<td>Putumayo, Colombia</td>
<td>(Mideros et al., 2016)</td>
</tr>
<tr>
<td>P8077</td>
<td>Solanum betaceum</td>
<td>P.betacei</td>
<td>Putumayo, Colombia</td>
<td>(Mideros et al., 2016)</td>
</tr>
<tr>
<td>P8093</td>
<td>Solanum betaceum</td>
<td>P.betacei</td>
<td>Putumayo, Colombia</td>
<td>(Mideros et al., 2016)</td>
</tr>
<tr>
<td>P9151</td>
<td>Solanum betaceum</td>
<td>P.betacei</td>
<td>Putumayo, Colombia</td>
<td>(Mideros et al., 2016)</td>
</tr>
<tr>
<td>P9153</td>
<td>Solanum betaceum</td>
<td>P.betacei</td>
<td>Putumayo, Colombia</td>
<td>(Mideros et al., 2016)</td>
</tr>
<tr>
<td>P8029</td>
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<td>P.betacei</td>
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<td>(Mideros et al., 2016)</td>
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<td>N9022</td>
<td>Solanum betaceum</td>
<td>P.betacei</td>
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<td>N9035</td>
<td>Solanum betaceum</td>
<td>P.betacei</td>
<td>Nariño, Colombia</td>
<td>(Mideros et al., 2016)</td>
</tr>
<tr>
<td>N9070</td>
<td>Solanum betaceum</td>
<td>P.betacei</td>
<td>Nariño, Colombia</td>
<td>(Mideros et al., 2016)</td>
</tr>
<tr>
<td>Sbc3#7</td>
<td>Solanum betaceum</td>
<td>P.betacei</td>
<td>Cundinamarca, Colombia</td>
<td>(Chaves et al, 2016)</td>
</tr>
<tr>
<td>VCP7#10</td>
<td>Solanum tuberosum</td>
<td>P.infestans</td>
<td>Cundinamarca, Colombia</td>
<td>(Chaves et al, 2016)</td>
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<tr>
<td>STT235</td>
<td>Solanum tuberosum</td>
<td>P.infestans</td>
<td>Nariño, Colombia</td>
<td>(Parra et al, 2016)</td>
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<tr>
<td>Z-32</td>
<td>Solanum phureja</td>
<td>P.infestans</td>
<td>Zipaquirá, Colombia</td>
<td>(Cárdenas et al, 2012)</td>
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Supplementary Table 2. Sequence of specific qRT-PCR oligonucleotides used to assess marker gene expression during the infection cycle of *P. betacei* and *P. infestans* on tree tomato.

<table>
<thead>
<tr>
<th>Gene ID</th>
<th>Gene name</th>
<th>Fragment size (bp)</th>
<th>Oligonucleotide forward sequence (5’-3’)</th>
<th>Oligonucleotide reverse sequence (5’-3’)</th>
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<tbody>
<tr>
<td>ActA</td>
<td>Actin A</td>
<td>69</td>
<td>CATCAAGGAGAAGCTGA CGTACAT</td>
<td>GACGACTCGGCGGAGAGAT</td>
</tr>
<tr>
<td>Cdc14</td>
<td>Cell cycle regulator</td>
<td>88</td>
<td>GCACTTTAACTTGACTA TTCTTGA</td>
<td>CAAACGTCCTTAGTGAGATG</td>
</tr>
<tr>
<td>Hmp1</td>
<td>Haustorial-membrane protein</td>
<td>76</td>
<td>GGAAACTAGTTCCTTGC GTAACTTGCCCTC</td>
<td>GGAAGCGGCGCTCAGCTGCT AAGCCTCGAGCATG</td>
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