Expression of the GAF Sensor, Carbohydrate-Active Enzymes, Elicitins, and RXLRs Differs Markedly Between Two *Phytophthora cactorum* Isolates

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ABSTRACT

The phytopathogen *Phytophthora cactorum* infects economically important herbaceous and woody plant species. *P. cactorum* isolates differ in host specificity; for example, strawberry crown rot is often caused by a specialized pathotype. Here we compared the transcriptomes of two *P. cactorum* isolates that differ in their virulence to garden strawberry (Pc407: high virulence; Pc440: low virulence). De novo transcriptome assembly and clustering of contigs resulted in 19,372 gene clusters. Two days after inoculation of *Fragaria vesca* roots, 3,995 genes were differently expressed between the *P. cactorum* isolates. One of the genes that were highly expressed only in Pc407 encodes a GAF sensor protein potentially involved in membrane trafficking processes. Two days after inoculation, elicitors were highly expressed in Pc407 and

lipid catabolism appeared to be more active than in Pc440. Of the carbohydrate-active enzymes, those that degrade pectin were often more highly expressed in Pc440, whereas members of glycosyl hydrolase family 1, potentially involved in the metabolism of glycosylated secondary metabolites, were more highly expressed in Pc407 at the time point studied. Differences were also observed among the RXLR effectors: Pc407 appears to rely on a smaller set of key RXLR effectors, whereas Pc440 expresses a greater number of RXLRs. This study is the first step toward improving understanding of the molecular basis of differences in the virulence of *P. cactorum* isolates. Identification of the key effectors is important, as it enables effector-assisted breeding strategies toward crown rot-resistant strawberry cultivars.

Phytophthora cactorum (Lebert & Cohn) J. Schröt is the causative agent of rots, cankers, blights, and wilt diseases in more than 200 plant species (Erwin and Ribeiro 1996). The susceptible species include many economically important fruit trees such as apple, pear, peach, plum, and cherry and woody ornamentals such as rhododendron. In particular, *P. cactorum* limits the productivity of strawberry (*Fragaria × ananassa*) by causing crown rot and leather rot (Maas 1998). Leather rot targets immature and mature fruits, whereas crown rot appears on the crown and root tissues. Rotting disrupts the vascular system of the plant, leading to stunting or wilting. In severe infections, the whole plant may collapse within days.

According to pathogenicity trials combined with amplified fragment length polymorphism analysis, crown rot of strawberry is caused by a specialized *P. cactorum* pathotype in many parts of the world, whereas leather rot can be caused by various isolates representing different pathotypes (Eikemo et al. 2004). Strawberry crown rot isolates collected from several European countries, Australia, Japan, New Zealand, and South Africa are genetically very similar, but are distinct from the isolates of other hosts and those causing leather rot (Eikemo et al. 2004). In contrast, isolates collected from strawberries in the United States display higher

diversity, suggesting that *P. cactorum* might originate from North America (Eikemo et al. 2004; Hantula et al. 2000; Huang et al. 2004). In pathogenicity tests, strawberry isolates from the United States show intermediate virulence, whereas all other strawberry crown rot isolates are very aggressive toward strawberries (Eikemo et al. 2004; Hantula et al. 2000). In contrast, none of the isolates from other host plants cause crown rot symptoms in strawberry. A tendency to host specialization seems to also apply to other *P. cactorum* pathotypes. For example, isolates from birch and apple are usually able to cause more severe symptoms in their original hosts compared with isolates from other sources (Hantula et al. 2000; Van Der Scheer 1971).

The capability of a pathogen to cause infection depends on its ability to overcome the physical barriers (e.g., cell wall, cuticle/suberin layers), to suppress or circumvent the defense responses, and to acquire nutrients from the host. To achieve this, the pathogens secrete hydrolytic enzymes and effector proteins that interfere with the plant processes and depress immunity. Cell wall-degrading enzymes (CWDEs) include various carbohydrate-active enzymes (CAZymes), such as glycoside hydrolases (GHs), carbohydrate esterases (CEs), and polysaccharide lyases (PLs), that target plant cell wall polysaccharides and provide nutrients for the pathogen (Brouwer et al. 2014). The *Phytophthora* species secrete apoplastic elicitors, which serve as sterol carriers scavenging sterols from the plasma membranes, but also elicit hypersensitive cell death in plants (Derevnina et al. 2016; Vauthrin et al. 1999). Members of the necrosis- and ethylene-inducing peptide 1-like protein (NLP) and secreted cysteine-rich/*P. cactorum*-*Fragaria* (SCR/PcF) families are considered as extracellular toxins often triggering cell death in plant tissues, even though they may have other important functions as well (Chen et al. 2016; Dong et al. 2012). The RXLRs and Crinklers (CRNs) are *Phytophthora* effector classes known to be targeted to the inside of the plant cell (Anderson et al. 2015; Haas et al. 2009). Some members of these protein families can suppress hypersensitive cell death, whereas others induce cell death in the host plant (Amaro et al. 2017; Wang et al. 2011).

The *P. cactorum* effectors have been studied using an RNA sequencing (RNA-seq) approach (Chen et al. 2014, 2018). Chen

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et al. (2014) sequenced two pooled *P. cactorum* (isolate 10300) samples containing RNA from mycelia, sporangia, zoospores, cysts, and germinating cysts and assembled 21,662 unigenes. They identified 620 genes encoding pathogenesis-related proteins, which include 94 RXLRs, 64 CRNs, 44 elicitors or elicitor-like proteins, 31 NLPs, and three PcF-like proteins. In addition, Chen et al. (2018) analyzed the transcriptomes of *P. cactorum* (isolate 10300) mycelia, zoospores, and germinating cysts, identifying 166 effector genes. The genome of isolate 10300, which originates from an infected strawberry, was sequenced recently (Armitage et al. 2018). A wide arsenal of effectors was identified, including nearly 200 RXLRs. The genomes of two other *P. cactorum* isolates were sequenced recently: isolate LV007 from European beech (*Fagus sylvatica*) (Grenville-Briggs et al. 2017) and an isolate from *Panax ginseng* (Yang et al. 2018).

We have analyzed the transcriptional responses triggered by *P. cactorum* in *F. vesca* roots (Toljamo et al. 2016). The infection causes massive changes in the transcriptome, including reprogramming of the receptor-like kinase-based surveillance system and the genes involved in the biosynthesis of hormones and secondary metabolites, as well as down-regulation of genes implicated in cell wall biosynthesis. We also observed the expression of potential resistance genes within the *Resistance to Phytophthora cactorum* 1 locus identified by Davik et al. (2015) in *F. vesca*. In garden strawberry, *P. cactorum* resistance is known to be a quantitative, polygenic trait (Denoyes-Rothan et al. 2004; Shaw et al. 2006, 2008), and several quantitative trait loci controlling this trait were recently identified (Mangandi et al. 2017; Nellist et al. 2018). However, the genes and mechanisms behind the resistance are unknown and their identification requires substantial efforts. More profound understanding of *P. cactorum*–strawberry interaction is thus needed.

The aim of the present study was to compare the transcriptomes of two *P. cactorum* isolates (Pc407 and Pc440), which differ in their virulence to garden strawberry. In pathogenicity trials, Pc407 was shown to be very aggressive to garden strawberry, deteriorating or destroying 80% of the inoculated plants (cultivar Jonsok), whereas Pc440 caused only moderate symptoms in 10% of the plants (Rytönen et al. 2012). In contrast, Pc440 was more pathogenic to rhododendron (Rytönen et al. 2012). Hence, the objective here was to investigate the molecular basis underlying the virulence differences of these two *P. cactorum* isolates and to search for candidate genes that might contribute to the virulence.

MATERIALS AND METHODS

Cultivation of *P. cactorum*. The *P. cactorum* isolates, Pc407 and Pc440, were maintained on potato dextrose agar (Difco) at room temperature. To induce sporangia and zoospore production, the isolates were grown in pea broth (Zentmyer and Chen 1969) and soil extract water (Hamm and Hansen 1991) as previously described (Toljamo et al. 2016). Briefly, the isolates were grown for 3 to 5 days in 10 ml of pea broth at room temperature. The cultures were washed three times in sterile water and 10 ml of soil extract water was added. After incubation overnight under fluorescent light, the soil extract water was removed and the cultures were incubated 1 to 3 days under light. To induce zoospore production, cold (4°C) sterile water was added, and the cultures were kept at 4°C for 30 min before returning to room temperature. The zoospores were counted and the concentrations were adjusted with sterile water.

Virulence tests. The virulence tests were performed using whole *F. × ananassa* ‘Senga Sengana’ and *F. vesca* (clone Hawaii4.4 of the accession Hawaii4) plants. The origin and maintenance of the plants is described in Toljamo et al. (2016). Micro-propagated strawberries were planted in 10 × 10 × 11-cm pots in peat-sand mixture (3:1) and were inoculated four times with 5 ml of Pc407 and Pc440 zoospore suspensions 21, 29, 32, and 43 days after potting, respectively. The concentrations were 47,500,

130,000, 49,000, and 41,000 zoospores/ml, respectively. The plant biomass ($n = 8$ for *F. vesca* control, $n = 10$ for other groups) was analyzed 57 days after the first inoculation. Statistical analysis was performed in SPSS software. A one-way analysis of variance (ANOVA) was used to compare the biomasses of *F. vesca*, and the one-way Welch ANOVA and Games-Howell test were used for *F. × ananassa*.

The in vitro virulence tests were conducted using detached leaflets of *F. × ananassa* ‘Polka’ ($n = 12$), *F. vesca* Hawaii4.4 ($n = 12$), and leaves of *Rhododendron* ‘Eino’ ($n = 12$) and ‘Raisa’ ($n = 12$) as described in Toljamo et al. (2017). The leaflets and leaves were placed adaxial side up on moisturized polyester cloths in plastic boxes. Two holes were punched in the primary vein of each leaf/leaflet; at least 3 cm apart from each other. The clumps of liquid-grown mycelia (from 5-day-old pea broth cultures) were placed on top of the holes; Pc407 mycelia on one hole and Pc440 on the other. The leaflets and leaves were imaged and the areas of necrotic lesions were measured using the Fiji image processing package 5 days after inoculation. An exact sign test was used to compare the virulence differences of the isolates in SPSS software.

RNA-seq. Hydroponically grown *F. vesca* Hawaii4.4 plants were inoculated by immersing the roots in zoospore suspension (390 ml, 5,000 zoospores/ml) for 30 min as described by Toljamo et al. (2016). Two days after inoculation, the roots were collected (three biological replicates, each from a different container) and stored at –80°C. The samples were homogenized in grinding buffer (100 µl of buffer/10 mg of sample) containing 0.14 M of NaCl, 2 mM of KCl, 2 mM of KH₂PO₄, 8 mM of Na₂HPO₄ × 2 H₂O, 0.05% (vol/vol) Tween-20, 2% (wt/vol) polyvinylpyrrolidone 40, and 0.7% (wt/vol) bovine serum albumin (Thompson et al. 2003). RNA was isolated with the RNeasy Mini Kit (Qiagen) as described by Toljamo et al. (2016). In the following sections, these samples are referred to as Pc407Fv1-3 and Pc440Fv1-3 (Fig. 1).

In addition to the inoculated root samples, RNA was extracted from 5-day-old liquid cultures (peptone-glucose PG1 medium, Unestam 1965) of the Pc407 and Pc440 isolates. The mycelia samples (~30 mg) were harvested for extraction by tweezers. Tripure isolation reagent (Roche Life Science) (1 ml) was added to the mycelia, which were then homogenized in liquid nitrogen. The thawed samples were incubated at room temperature for 5 min and extracted with 200 µl of chloroform according to the manufacturer’s instructions. The aqueous phase was transferred to a new tube, and 500 µl of isopropanol was added. The samples were transferred to an RNeasy spin column and purification was continued using an RNeasy Mini Kit (Qiagen) according to the manufacturer’s instructions. In the following sections, these mycelia samples are referred to as Pc407M and Pc440M (Fig. 1).

Library construction and RNA-seq (101 bp, paired-end reads) of the inoculated root and mycelia samples were performed with

Fig. 1. Pipeline of data analysis. Pc407M and Pc440M are mycelium samples. Pc407Fv1-3 and Pc440Fv1-3 are *Phytophthora cactorum* samples from inoculated *Fragaria vesca* roots.

an Illumina HiSeq2000 sequencing system at the Weill Cornell Medical College Genomics Resources Core Facility, as described by Toljamo et al. (2016). The reads are available in the National Center for Biotechnology Information Sequence Read Archive (accession numbers SRR3743196, SRR3743197, SRR374198, SRR5894894, SRR5894895, SRR5894896, SRR5894897, and SRR5894898).

Data analysis. The data analysis steps are shown in Figure 1. Trimming and filtering of reads and mapping against the nuclear (version 1.0) and chloroplast genome of *F. vesca* were performed as described by Toljamo et al. (2016). In the mapping process, unmapped reads were automatically extracted to separate files. De novo assemblies with Trinity were made separately for Pc407 and Pc440 isolates using paired unmapped reads from the inoculation experiment (samples Pc407Fv1-3 and Pc440Fv1-3) and paired, filtered reads obtained from mycelia samples (Pc407M and Pc440M) (Haas et al. 2013).

Blastx search of contigs was made against the nr database using an e-value cutoff of 10^{-5} . Contigs that had the best blast hit with oomycete species were considered to represent *P. cactorum* genes. These oomycete contigs were extracted from the assemblies, and reads (unmapped reads from Pc407Fv1-3 and Pc440Fv1-3 samples and all trimmed reads from Pc407M and Pc440M samples) were mapped against these using Bowtie with default settings, except that all valid alignments per read pair were reported (–all option) (Langmead et al. 2009). Contigs representing the same gene were subsequently clustered using Corset (Davidson and Oshlack 2014). Default settings were used in the Corset run, but the threshold of the log likelihood ratio (–D option) was set very high (1E+12) to switch off the contig ratio test and to ensure that corresponding contigs from two isolates were clustered together. Normalized expression levels (counts per million [cpm] mapped reads) were calculated and differential expression analysis was performed for Pc407Fv and Pc440Fv samples with edgeR using the exact test (Robinson et al. 2010). Clusters were considered to be differentially expressed if the false discovery rate (FDR) was < 0.05 , and the $\log_2$ fold-change ($\log_2FC$) was < -1 or > 1 . The completeness of transcriptomes was assessed with BUSCO version 2.0.1 using the *alveolata_stramenophiles_ensembl* lineage data set (Simão et al. 2015).

Peptides (>30 amino acids) were derived from contigs using TransDecoder (Haas et al. 2013). Contigs and proteins were annotated and Gene Ontology (GO) terms were searched using Blast2GO, PANNZER, and InterProScan (Conesa et al. 2005; Jones et al. 2014; Koskinen et al. 2015). GO term enrichment analysis was made with the BiNGO plug-in in Cytoscape (Maere et al. 2005). Effectors were identified based on descriptions of Blast2GO and PANNZER annotations, using blastp searches (e-value cutoff 10^{-5}) against known effector sequences from the nr database, and by performing an RXLR motif search. The presence of signal peptides was tested with SignalP 3.0 (Bendtsen et al. 2004). LOCALIZER was used to predict the subcellular localization of RXLR effectors in planta (Sperschneider et al. 2017). These predictions were made for sequences that were clipped after the RXLR motif. CAZymes were searched using the Database for Carbohydrate-Active Enzyme Annotation 2 (dbCAN2) with default settings (Yin et al. 2012; Zhang et al. 2018). CAZyme annotations were accepted if two of the three tools (Hotpep, DIAMOND, or HMMER) gave the same results, as recommended by the authors.

Polymerase chain reaction analysis of GAF sensor and RXLR effectors. Polymerase chain reaction (PCR) analysis was conducted to examine the presence of the GAF sensor (Cluster-4332.0) and three RXLR effectors (Cluster-8163.0, Cluster-1816.0, and Cluster-1783.0) in the genomes of *P. cactorum* isolates Pc407 and Pc440, as well as from four other isolates (GNR3, LPV2, RVO1, and VST1) from crown rot-diseased strawberries, available in our culture collection. These genes were chosen for PCR because they were very highly expressed in either Pc407 or Pc440 and were

absent or nearly absent from the other isolate. DNA was extracted from liquid culture-grown mycelia with the E.Z.N.A. Fungal DNA Mini Kit (Omega Bio-tek). The primers designed for contigs with Primer3 (Untergasser et al. 2012) are shown in Supplementary Table S1. The reaction mixture contained DreamTaq Green PCR Master Mix (Thermo Fisher Scientific), 0.25 μ M of forward and reverse primer, and 1 μ l of the DNA template in the final volume of 20 μ l. The cycling program was as follows: initial denaturation at 95°C for 2 min, followed by 30 cycles of 95°C for 30 s, 60°C for 30 s, 72°C for 30 s, and final elongation at 72°C for 10 min. PCR products were separated on 1.5% agarose gel and visualized with ethidium bromide.

Quantitative reverse transcription PCR analysis. To validate the RNA-seq results and to obtain more information on the expression patterns of the most interesting genes, a quantitative reverse transcription (qRT)-PCR analysis was conducted for five genes: elicitor (ELI; Cluster-11371.0), GAF sensor (Cluster-4332.0), GH1 (Cluster-10478.0), and two RXLR effectors (Cluster-3979.0 and Cluster-8163.0). Hydroponically grown micropropagated Senga Sengana plants were inoculated with Pc407 and Pc440 zoospores (390 ml, 5,000 zoospores/ml) for 30 min and root samples were collected 48 h after inoculation. Roots of H4.4 plants were inoculated for 2 h with zoospore suspensions (500 zoospores/ml) and samples were collected 20, 48, and 120 h after inoculation. Roots were ground in liquid nitrogen and 50 μ l of grinding buffer was added per 10 mg of plant material. RNA extraction was performed with the RNeasy Plant Kit (Qiagen), DNase treatment was conducted with the RNase-free DNase set (Qiagen), and the RNA concentration was determined with a NanoDrop spectrophotometer. For the cDNA synthesis, 50 to 100 ng of total RNA was used and synthesis was performed with the Verso cDNA synthesis kit (Thermo Fisher Scientific).

The gene encoding ubiquitin-conjugating enzyme E2-16 kDa (Cluster-12441.0) was used as an internal control (Yan and Liou 2006). The reaction mixture (10 μ l) contained LightCycler 480 SYBR Green I Master Mix (Roche), 0.4 μ M of forward and reverse primer, and 4 μ l of the 1:5 diluted cDNA template. Three technical replicates from each sample were analyzed. qRT-PCR was performed with a Roche Lightcycler 480 using the following program: initial denaturation at 95°C for 10 min, followed by 45 cycles of 95°C for 20 s, 58°C for 20 s, 72°C for 20 s, and final elongation at 72°C for 5 min.

RESULTS

Virulence tests. To compare the virulence of the two *P. cactorum* isolates, Pc407 and Pc440, inoculation tests with detached leaves and whole plants were conducted (Fig. 2; Table 1). According to the in vitro leaf tests, Pc407 was more virulent than Pc440 to garden strawberry Polka, whereas Pc440 caused larger lesions in the rhododendron leaves. In the leaflets of *F. vesca* Hawaii4 (clone H4.4), no statistically significant differences were found between the *P. cactorum* isolates. Similar results were obtained with the whole plants (Fig. 2). Because no necrotic symptoms were observed in the crown tissue, the virulence was estimated by measuring the aboveground biomass of the plants. *P. cactorum* isolates did not reduce the *F. vesca* biomass, whereas Senga Sengana plants inoculated with Pc407 were severely stunted. The biomass of Pc440-inoculated Senga Sengana plants did not differ from the control plants.

Overview of de novo transcriptome assemblies of *P. cactorum* isolates. The transcriptomes of the two *P. cactorum* isolates (Pc407: high virulence; Pc440: low virulence to garden strawberry) were assembled using reads derived from liquid culture-grown mycelia and the unmapped reads of the dual transcriptome samples (i.e., the reads that did not map to the *F. vesca* genome) (Fig. 1; Supplementary Table S2). A total of 67,318 and 76,541 contigs were assembled from all Pc407 and Pc440 samples, 19,867 and

25,095 of which were of oomycete origin according to blastx results, respectively. Table 2 shows an overview of the *P. cactorum* contigs and a summary of the BUSCO analysis.

Clustering of the contigs that represent the same gene in Pc407 and Pc440 transcriptomes resulted in 19,372 clusters, with the number of contigs in each cluster varying from 1 to 29 (Supplementary Table S3). There was some variation in the percentage of *P. cactorum* reads in the dual transcriptome samples: of the filtered reads, ~4, 10, and 20% were derived from *P. cactorum* in Pc407Fv replicates and 2, 4, and 10% in Pc440Fv replicates. The biological replicates clustered close to each other in the multidimensional scaling plot (Fig. 3).

Differential in planta expression of the genes of *P. cactorum* isolates. In differential expression analysis, 3,955 clusters (~20%) were significantly differentially expressed ($\log_2FC < -1$ or > 1 , $FDR < 0.05$) between the isolates in planta, of which 1,960 and 1,995 were more highly expressed in Pc407Fv and Pc440Fv, respectively. The cluster that was most significantly expressed at a higher level in Pc407Fv ($\log_2FC = -10.06$, $FDR = 8.09E-128$) encoded a 757 amino-acid GAF sensor protein (Cluster-4332.0). It was very highly expressed in Pc407Fv samples (744 to 874 cpm) and was also present in Pc407M (62 cpm), but it had very low expression in Pc440Fv and Pc440M samples (0 to 2 cpm). According to the PCR results, the gene encoding the GAF sensor was present in the genome of both isolates (Supplementary Fig. S1). InterProScan and hmmscan analyses (Finn et al. 2015) indicated the FYVE zinc finger domain (PF01363) in the middle of the protein and the GAF domain in the C terminus (PF01590). In addition, the InterProScan sequence search tool showed similarity to chaperonin (PTHR11353, Panther Classification System). The FYVE zinc finger domain contained eight conserved cysteine residues (involved in zinc/metal binding); however, because some of the critical amino acids of the phosphatidylinositol 3-phosphate binding motifs were missing from this domain, it seems to fall into the category of

FYVE-like domains. The most significantly differentially expressed clusters also contained several hypothetical proteins and clusters related to (retro)transposons in both isolates. In addition, many CAZymes and effectors were differentially expressed between the isolates and will be discussed below.

GO term enrichment analysis and the genes within the GO terms. GO term enrichment analysis revealed 111 and 336 biological processes that were enriched among the gene sets that were significantly more highly expressed in Pc407Fv and Pc440Fv, respectively (Supplementary Table S4). Among the gene set that was more highly expressed in Pc407Fv, several enriched GO terms were related to lipid catabolism. These included many genes involved in β -oxidation of fatty acids, such as those encoding long-chain fatty acid-coenzyme A (CoA) ligases, acyl-CoA oxidases, acyl-CoA dehydrogenases, and 3,2-transenoyl-CoA isomerases. Genes encoding glucosylceramidases and alkaline ceramidases involved in sphingolipid catabolism, and lipases catalyzing lipid hydrolysis, were also more highly expressed in Pc407Fv. In addition, sphingolipid transporter (Protein spinster homolog 1) and fatty acid transporter (solute carrier family 27), responsible for the uptake of long-chain fatty acids, showed higher expression in Pc407Fv. Furthermore, processes related to cellular membrane fusion, vesicle organization, and transport were among the enriched biological processes in Pc407Fv. Genes involved in these processes included several FYVE zinc finger domain and/or START-like domain-containing proteins, SNARE proteins, Sec1-like proteins, and sorting nexin. In contrast, several processes related to ribosome biogenesis, gene expression, translation, amino acid biosynthesis, and pectin catabolic process were enriched among the genes that were more highly expressed in Pc440Fv.

CAZymes. The dbCAN2 search identified 326 and 333 potential CAZymes from Pc407 and Pc440 protein sets, respectively, derived from 297 gene clusters. Interesting expression differences between the isolates were found among several

Fig. 2. Virulence tests on whole plants. **A,** *Fragaria* × *ananassa* ‘Senga Sengana’. **B,** *F. vesca* (H4.4). One-way Welch analysis of variance (ANOVA) [$F(2,15.414) = 42.912$, $P = 0.000001$] and Games-Howell post hoc analysis showed significant differences between the *F. × ananassa* groups: the biomasses of Pc407-inoculated plants were significantly lower compared with the control (−24.28; 95% confidence interval [CI], −30.674 to −17.886) and Pc440-inoculated plants (−22.39; 95% CI, −28.784 to −15.996). According to one-way ANOVA [$F(2,25) = 2.4$, $P = 0.111$], there were no significant differences between *F. vesca* groups.

TABLE 1. Virulence tests on detached leaves/leaflets²

Plant/cultivar or accession	n	Pc407 lesion area (mm ²)	Pc440 lesion area (mm ²)	Median difference (mm ²)	Negative/positive differences	P value
<i>Fragaria</i> × <i>ananassa</i> ‘Polka’	12	72.4 ± 21.6	2.9 ± 1.0	49.6	12/0	<0.001
<i>F. vesca</i> Hawaii4.4	12	2.5 ± 0.6	4.6 ± 1.0	−1.3	4/8	0.388
<i>Rhododendron</i> ‘Eino’	12	49.8 ± 4.1	478.9 ± 40.1	−371.1	0/12	<0.001
<i>Rhododendron</i> ‘Raisa’	12	38.0 ± 5.7	299.3 ± 22.9	−265.2	0/12	<0.001

² An exact sign test was used to compare the differences in lesion areas caused by Pc407 and Pc440 isolates. Data for Pc407 and Pc440 are presented as the mean ± standard error.

CAZyme families. For example, all four CE5 clusters encoding cutinases were more highly expressed in Pc407Fv, whereas 11 CE8 clusters encoding pectinesterases showed higher expression in Pc440Fv. Several PL clusters were also more highly expressed in Pc440Fv, including five pectin lyases (PL1 family), four pectate lyases (PL3), and one rhamnogalacturonase cluster (PL4), all of which are involved in pectin degradation. Of the GH families, four pectin-degrading polygalacturonases (GH28) and six arabinan endo-1,5- α -L-arabinosidases (GH43) showed higher expression in Pc440Fv, as did six members of GH12 family encoding Cell 12A endoglucanases. In contrast, members of the GH1, GH3, and GH30 families were often more highly expressed in Pc407Fv. Most of the members of the GH1 family were annotated as Prunasin hydrolases by PANNZER, whereas the members of GH30 were glucosylceramidases.

RXLR effectors. The most abundant effector group encoded by the *P. cactorum* transcriptomes was RXLRs, with 158 clusters (Table 3). Approximately 80% of these effectors contained the canonical RXLR sequence. In one case (Cluster-5412.0), Pc407 and Pc440 proteins possessed different motifs, with Pc440 having the RLLR and Pc407 having the RLLW sequence. Protein domains identified in RXLR candidates included the NUDIX hydrolase domain, tetratricopeptide repeat, and ABC1 family/protein kinase-like domain. According to LOCALIZER analysis, 29 RXLRs possessed a nuclear localization signal (NLS), whereas two effectors might be targeted to mitochondria. In addition, one RXLR protein was predicted to possess both mitochondrial transit peptide and NLS. This cluster was significantly more highly expressed in Pc407. Of the nucleus-targeted RXLRs, ~80% were differentially expressed between the isolates.

One of the most interesting RXLR effectors (Cluster-8163.0) was identical to PcRXLR21 protein reported by Chen et al. (2014) and was predicted by LOCALIZER as being targeted to the nucleus in planta (Sperschneider et al. 2017). This cluster was highly expressed in Pc407Fv (266 to 367 cpm) and in Pc407M (538 cpm) compared with Pc440Fv and Pc440M samples (<1 cpm). The presence of this gene in both isolates was confirmed by PCR. In total, 10 RXLR clusters had an expression level >100 cpm in Pc407Fv, whereas Pc440Fv contained as many as 20 of such clusters. In particular, RXLR Cluster-3979.0 showed very high expression (2,337 to 2,931 cpm) in Pc440Fv, but only minor expression (<1 cpm) in Pc407Fv. In addition, transcripts of 28 RXLRs clusters were found exclusively in Pc440. The absence of the two most highly expressed Pc440-specific RXLRs (Cluster-1783.0 and Cluster-1816.0) from the genome of Pc407 was confirmed by PCR. No PCR products of these RXLR genes were detected in Pc407 or in other crown rot-type *P. cactorum* isolates tested (GNR3, LPV2, RVO1, and VST1).

Other effectors. The most highly expressed cluster in all Pc407Fv samples encoded elicitin (mean 13,229 cpm), and it was also the most highly expressed cluster in Pc407M (mean 19,008

cpm) and Pc440M (mean 11,149 cpm) samples. This elicitin was named PcINF1 because of its homology to *P. infestans* INF1 (Chen et al. 2018). Of the 62 elicitin clusters, 14 were significantly more highly expressed in Pc407Fv, whereas nine showed higher expression in Pc440Fv. In addition, the total expression level of elicitin clusters was higher in Pc407Fv (mean 28,429 cpm) compared with Pc440Fv (mean 15,020 cpm). In contrast, NLP clusters showed higher overall expression in Pc440Fv (mean 1,453 cpm) compared with Pc407Fv (507 cpm). Eight NLP clusters were more highly expressed in Pc440Fv, four of which had a mean expression level > 100 cpm. In particular, two NLPs showed high expression (>200 cpm) in Pc440Fv but were nearly absent in Pc407Fv samples (<1 cpm). In Pc407Fv, nine NLP clusters were significantly more highly expressed compared with Pc440Fv, but all of these clusters had rather low expression levels (<50 cpm). Of the PcF/SCR family, clusters encoding PcF, SCR96, and SCR121 were highly expressed in both isolates, whereas six clusters showed very low expression (<2 cpm). The most highly expressed SCR cluster encoded SCR108-like protein that contained cellulose-binding module 1 in the C terminus. This cluster was significantly more highly expressed in the highly virulent Pc407Fv (>2,000 cpm) compared with Pc440Fv (>800 cpm).

Validation of the RNA-seq data by qRT-PCR. The expression patterns of the five most interesting *P. cactorum* genes were explored at three time points (20, 48, and 120 h after inoculation) during the interaction with *F. vesca* roots. In addition, the

Fig. 3. Multidimensional scaling plot, in which distances between pairs of samples correspond to biological coefficient of variation (BCV) calculated from 500 genes having the largest biological variation between the samples. Isolates are separated from each other in dimension 1, whereas mycelia and in planta samples are separated in dimension 2.

TABLE 2. Summary of de novo assembled transcriptomes of Pc407 and Pc440 isolates and results of BUSCO analysis

Feature	Pc407	Pc440
<i>P. cactorum</i> contigs, <i>n</i>	19,867	25,095
Contig length, bp		
Maximum	20,265	15,388
Minimum	201	201
Median	1,252	1,108
Average	1,685	1,434
Contig N50, bp	2,541	2,097
BUSCOs, <i>n</i> (%)		
Complete	228/234 (97.4)	223/234 (95.3)
Fragmented	2/234 (0.9)	2/234 (0.9)
Missing	4/234 (1.7)	9/234 (3.8)
Open reading frames (>30 amino acids)	32,672	39,175

TABLE 3. Number of effector proteins, clusters, and differentially expressed effector clusters^z

Effector group	Pc407 number of proteins	Pc440 number of proteins	Number of clusters	Higher expression in Pc407Fv	Higher expression in Pc440Fv
ELI and ELL	64	64	62	14	9
CRN-like	84	88	102	17	17
NLP	34	35	35	9	8
RXLR	103	137	158	22	68
SCR/PcF	13	14	13	4	2

^z ELI = elicitin, ELL = elicitin-like, CRN = crinkler, NLP = necrosis- and ethylene-inducing peptide 1-like protein, and SCR/PcF = secreted cysteine-rich/*Phytophthora cactorum*-Frugaria.

expression differences were studied 48 h after inoculation using Senga Sengana as a host plant. The delta cycle threshold (dCt) values ($Ct_{\text{Reference gene}} - Ct_{\text{Target gene}}$) of these genes in each sample are presented in Figure 4, and the fold differences (Livak and Schmittgen 2001) are provided in Supplementary Table S5. Because the gene expression levels in some of the biological replicates were too low to

be detected by qRT-PCR, it was not possible to calculate dCt values or to perform statistical analysis. However, it can be seen from the scatter plots that the expression differences of RXLR effectors and GAF sensor between the isolates were clear irrespective of the time point or of the host plant. GAF sensor and RXLR (Cluster-8163.0) were always expressed more highly in Pc407, whereas RXLR

Fig. 4. A quantitative reverse transcription polymerase chain reaction analysis of **A**, RXLR^a (Cluster-3979.0), **B**, RXLR^b (Cluster-8163.0), **C**, GAF sensor (Cluster-4332.0), **D**, elicitin (ELI; Cluster-11371.0), and **E**, glycosyl hydrolase 1 (GH1; Cluster-10478.0) in Pc407 and Pc440 isolates during the interaction with *Fragaria vesca* (Fv) Hawaii4.4 (20, 48, or 120 h after inoculation) and *F. x ananassa* (Fa) ‘Senga Sengana’ (48 h after inoculation). The delta cycle threshold (dCt) values ($Ct_{\text{Reference gene}} - Ct_{\text{Target gene}}$) of each replicate as well as the replicates with no detected (ND) Ct values are presented. **F**, Expression levels (in counts per million [cpm]) of the genes and the internal control gene, ubiquitin-conjugating enzyme (UBC; Cluster-12441.0), in RNA-sequenced samples of Pc407 and Pc440 in *F. vesca* Hawaii4.4 (48 h after inoculation).

(Cluster-3979.0) showed higher expression in Pc440, thus corroborating the results of the RNA-seq analysis. Elicitin and glycosyl hydrolase had more variation in their expression patterns. After 48 h of inoculation, the expression of these genes appeared to be higher in Pc407 in *F. vesca*, which is in line with the RNA-seq data. However, at 20 h and 120 h after inoculation, there were no clear differences between the isolates.

DISCUSSION

Strawberry crown rot is often caused by a specialized pathotype of *P. cactorum* (Eikemo et al. 2004). To better understand the molecular basis of the pathogenicity and virulence differences in *P. cactorum*, we sequenced the transcriptomes of two *P. cactorum* isolates. Of these, Pc407 was aggressive and Pc440 was weakly virulent to garden strawberry, whereas Pc440 was more virulent to rhododendron (Fig. 2; Table 1). In *F. vesca*, no significant virulence differences were found between the isolates (Fig. 2; Table 1). The results obtained in our virulence tests are in line with the studies conducted by Rytönen et al. (2012). For the RNA-seq experiment, the woodland strawberry (accession Hawaii4, clone H4.4) was chosen as a host plant, because the genome of *F. vesca* is sequenced and it is thereby a good model plant for molecular studies (Shulaev et al. 2011).

De novo assemblies of isolate-specific transcriptomes were constructed using the reads obtained from liquid culture-grown mycelia and inoculated root samples of *F. vesca* (2 days after inoculation) (Fig. 1). The number of genes (19,372 clusters) was fairly similar to the number of unigenes (21,662) obtained for *P. cactorum* transcriptome by Chen et al. (2014). A BUSCO analysis also indicated that the Pc407 and Pc440 transcriptome assemblies were quite complete (>95% complete BUSCOs) (Table 2). In sequenced *P. cactorum* genomes, the number of predicted protein-coding genes is 21,876 (Grenville-Briggs et al. 2017), 23,884 (Armitage et al. 2018), or 27,981 (Yang et al. 2018). In addition to *P. cactorum* contigs, a high number of *F. vesca* contigs were present in the assemblies. This is probably attributable to single nucleotide polymorphism differences between the inbred line of accession Hawaii 4 (H4×4) used in genome sequencing (Shulaev et al. 2011) and the Hawaii4 clone (H4.4) used in our study. Because of these differences, all *F. vesca* reads did not map to the reference genome and hence were included in the transcriptome assembly together with the genuine *P. cactorum* reads.

The infection level of *F. vesca* roots varied markedly between the biological replicates. A possible explanation is that the number of zoospores used for inoculation was not equal, as *Phytophthora* zoospores have a tendency to swim upward (Ochiai et al. 2011). However, this should not be considered as a weakness of the study; it rather confirms that the expression differences seen in this experiment are valid over a wide range of infection levels.

In the differential expression analysis, 3,955 clusters (20%) were significantly differentially expressed between the isolates in planta. The qRT-PCR analysis of five *P. cactorum* genes (at 48 h after inoculation) corroborated the RNA-seq results and indicated that the expression differences between the isolates also remained in the garden strawberry (Fig. 4). In the case of the GAF sensor and RXLRs, the expression differences were clear throughout the infection, whereas the expression patterns of elicitin and GH1 showed more fluctuation. It is thus important to realize that the expression levels vary during the interaction, whereas the RNA-seq data represent a single time point. Nevertheless, the differences seen in RNA-seq potentially represent genes that may have an effect on virulence differences and on the outcome of the interaction between the plant and the oomycete. The genes and gene groups differentially expressed between the isolates are discussed below.

RXLR effectors are known as key components in *Phytophthora*-plant interactions (Anderson et al. 2015). They can function as virulence or avirulence factors, often determining the outcome of

the interaction. In this study, 22 RXLR genes had significantly higher transcript levels in Pc407Fv than in Pc440Fv. A particular RXLR (Cluster-8163.0) showed high expression in Pc407Fv but very low expression in Pc440Fv. A more detailed analysis indicated that this gene is present in both isolates, but it was not expressed or had very low expression in all Pc440 samples, whereas the expression was high in Pc407 (20, 48, and 120 h after inoculation) (Fig. 4). Chen et al. (2014) showed that *P. cactorum* isolate 10300 expressed this gene (PcRXLR21) throughout the infection (1.5 to 96 h) in *Nicotiana benthamiana* roots as well as in germinating cysts, suggesting an important role in the infection process.

On the other hand, the expressed RXLR effector set was broader in Pc440Fv than in Pc407Fv. In total, 68 RXLR clusters showed significantly higher expression in Pc440Fv, with 28 of them being expressed exclusively in Pc440. Moreover, at least two RXLRs were completely missing from the genome of Pc407 as well as from the genomes of other crown rot-type *P. cactorum* isolates tested in this study. In addition, some NLP genes showed high expression levels in Pc440Fv but very low expression in Pc407Fv. The presence of these effectors in the transcriptome of low-virulent Pc440 isolate and their absence from the highly virulent Pc407 isolate raises a question of whether some of them function as avirulence genes in strawberry. Indeed, previous studies have shown that RXLRs may be the key factors determining the host range of the pathogen, as their recognition seems to play important roles in nonhost resistance (Lee et al. 2014; Vega-Arreguín et al. 2014). Conversely, the elimination, alteration, or suppression of critical avirulence genes by various mechanisms, such as transposon insertions, mutations, or epigenetic reprogramming, may enable the escape from host defense and lead to the gain of virulence (Na and Gijzen 2016). A recent example of this phenomenon is an asexual lineage of *P. infestans* (EC-1) that shows gene expression polymorphism between isolates (Pais et al. 2018). In one of the two studied isolates, expression of the RXLR effector gene *Avrnt1* was silenced, rendering this isolate virulent to a potato carrying the *RPI-vnt1.1* resistance gene (Pais et al. 2018).

The gene most significantly more highly expressed in Pc407Fv encodes a GAF sensor protein. The protein contains the FYVE-like zinc finger and GAF domains. This combination is the most abundant oomycete-specific domain bigram found in the domain-centric analysis of 67 eukaryotic species (Seidl et al. 2011). The GAF domain is named after the cGMP-specific phosphodiesterase, adenylyl cyclase, and FhlA proteins in which it is present. It is a ubiquitous domain found in many sensory and signaling proteins and acts like a small-molecule-binding molecular switch, regulating the activity of adenylyl and guanylyl cyclases and phosphodiesterases (Martinez et al. 2002). FYVE-like domains are able to bind phospholipids and many of the characterized proteins containing this domain appear to be involved in membrane trafficking events (Supplementary Data Set S1 and references therein). Therefore, it is possible that the GAF sensor protein also has some role in membrane trafficking. Based on GO term analysis, biological processes related to membrane fusion, vesicle organization, and transport processes were significantly enriched among the gene set that was more highly expressed in Pc407Fv. However, the role of these processes in pathogenesis is unknown. Therefore, it is not possible to draw further conclusions about their contribution to virulence differences. Yet GAF-FYVE bigram-containing proteins can be considered as interesting targets for future studies.

Several elicitors, including PcINF1 (Chen et al. 2018), showed very high expression in all Pc407Fv samples. The expression of elicitors varies during the life cycle, and they are abundantly expressed especially in mycelia (Jiang et al. 2006). *Phytophthora* spp. require sterols for growth and sporulation but are unable to synthesize them. Because elicitors serve as sterol carriers, they may have a positive effect on the fitness of *Phytophthora* pathogens. For example, *P. parasitica* progeny that produce detectable amounts of elicitors show higher growth rates in vitro compared

with nonproducers (Kamoun et al. 1994). Elicitin-producing *P. parasitica* progeny are, however, less virulent or nonvirulent to tobacco, because elicitors induce a hypersensitive response (HR) in *Nicotiana* species (Kamoun et al. 1994). In contrast, a positive correlation between elicitor expression, virulence, and sporulation has been observed in *P. ramorum* isolates representing three clonal lineages (Manter et al. 2010). Two of the three *P. ramorum* lineages expressed higher levels of Ram- α 2 elicitor and caused more prominent necrosis in rhododendron leaves, whereas the third lineage caused only small lesions. Thus, the effect of elicitors on virulence can be either negative or positive depending on the pathosystem. The *P. cactorum* PcINF1 elicitor is known to trigger HR cell death in solanaceous species (Chen et al. 2018), but its HR-inducing capability in strawberry is unknown.

In total, 297 potential CAZyme gene clusters were identified in *P. cactorum* transcriptomes. Of the CWDEs, members of the GH12 family (endoglucanases) (Costanzo et al. 2006) were more highly expressed in Pc440Fv. Several CAZymes involved in pectin degradation showed higher transcript levels in Pc440Fv as well. This is unexpected, since CWDEs are known to be important pathogenicity factors in phytopathogens. For example, pectolytic activity of *P. capsici* culture filtrates correlates positively with lesion severity (Jia et al. 2009). On the other hand, pectin degradation is considered to take place early in the infection process (Blackman et al. 2014). For example in *P. parasitica*, pectin-degrading CAZyme families CE8, GH28, and PL4 are predominantly expressed during early stages (30 to 36 h after inoculation) of lupine root infection (Blackman et al. 2015). Therefore, it is possible that higher expression of pectin-degrading enzymes in Pc440Fv reflects slower progression of the disease. Larger-scale RNA-seq analysis including several time points would reveal whether the gene expression dynamics is delayed in the Pc440 isolate compared with Pc407.

The majority of members of the GH1 family showed higher expression in Pc407Fv, as did 10 members of the GH3 family. Besides cell wall degradation, these CAZymes may play roles in the hydrolysis of glycosylated secondary metabolites. The ability to utilize and detoxify host plant defense compounds, ginsenosides, seems to be an important infection strategy for ginseng-infecting *P. cactorum* isolate. Yang et al. (2018) suggested that glycosyl hydrolases and detoxification-related enzymes might be responsible for this capability. Another ginseng root pathogen, *Pythium irregulare*, is known to employ a similar infection strategy. Three ginsenoside hydrolyzing β -glucosidases have been identified from *P. irregulare*, two of which seem to belong to the GH1 family (Neculai et al. 2009). Furthermore, the pathogenicity of *P. irregulare* isolates positively correlates with ginsenosidase activity (Ivanov and Bernards 2012). Indeed, it has been recognized for many years that the capability to tolerate and detoxify plant defense compounds can be an important virulence strategy for phytopathogens (VanEtten et al. 2001). To determine whether strawberry secondary metabolites and their detoxification play a role in *P. cactorum* host specificity, it might be worthwhile to compare the sensitivity of isolates to strawberry metabolite extracts.

In summary, RNA-seq revealed interesting differences in the transcriptomes of two *P. cactorum* isolates. In particular, the expressed RXLR sets differed markedly between Pc407Fv and Pc440Fv at the time point studied. Because RXLRs are known to have a crucial role in *Phytophthora*-plant interaction, they can also be considered as the most probable virulence determinants in this pathosystem. The RXLR genes that are constantly highly expressed exclusively in one of the isolates are the most promising targets for functional studies. In addition to RXLRs, expression differences were observed in CAZymes, elicitors, and genes involved in membrane trafficking and lipid catabolism. To better understand the role of these factors and to evaluate their possible contribution to virulence differences, RNA-seq analysis should be extended to comprise more time points and multiple *P. cactorum* isolates. In this respect, a genetic approach employing the progeny of

Pc407 and Pc440 isolates could be particularly useful. Identification of key effectors and improved understanding of the infection mechanisms are vital for advanced breeding strategies that aim at crown rot-resistant strawberry cultivars.

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LITERATURE CITED

- Amaro, T. M., Thilliez, G. J., Motion, G. B., and Huitema, E. 2017. A perspective on CRN proteins in the genomics age: Evolution, classification, delivery and function revisited. *Front. Plant Sci.* 8:99.
- Anderson, R. G., Deb, D., Fedkenheuer, K., and McDowell, J. M. 2015. Recent progress in RXLR effector research. *Mol. Plant-Microbe Interact.* 28: 1063-1072.
- Armitage, A. D., Lysøe, E., Nellist, C. F., Lewis, L., Cano, L. M., Harrison, R. J., and Brurberg, M. B. 2018. Bioinformatic characterisation of the effector repertoire of the strawberry pathogen *Phytophthora cactorum*. *PLoS One* 13:e0202305.
- Bendtsen, J. D., Nielsen, H., von Heijne, G., and Brunak, S. 2004. Improved prediction of signal peptides: SignalP 3.0. *J. Mol. Biol.* 340:783-795.
- Blackman, L. M., Cullerne, D. P., and Hardham, A. R. 2014. Bioinformatic characterisation of genes encoding cell wall degrading enzymes in the *Phytophthora parasitica* genome. *BMC Genomics* 15:785.
- Blackman, L. M., Cullerne, D. P., Torreña, P., Taylor, J., and Hardham, A. R. 2015. RNA-seq analysis of the expression of genes encoding cell wall degrading enzymes during infection of lupin (*Lupinus angustifolius*) by *Phytophthora parasitica*. *PLoS One* 10:e0136899.
- Brouwer, H., Coutinho, P. M., Henrissat, B., and de Vries, R. P. 2014. Carbohydrate-related enzymes of important *Phytophthora* plant pathogens. *Fungal Genet. Biol.* 72:192-200.
- Chen, X.-R., Huang, S.-X., Zhang, Y., Sheng, G.-L., Zhang, B.-Y., Li, Q.-Y., Zhu, F., and Xu, J. Y. 2018. Transcription profiling and identification of infection-related genes in *Phytophthora cactorum*. *Mol. Genet. Genomics* 293:541-555.
- Chen, X.-R., Li, Y.-P., Li, Q.-Y., Xing, Y.-P., Liu, B.-B., Tong, Y.-H., and Xu, J.-Y. 2016. SCR96, a small cysteine-rich secretory protein of *Phytophthora cactorum*, can trigger cell death in the Solanaceae and is important for pathogenicity and oxidative stress tolerance. *Mol. Plant Pathol.* 17:577-587.
- Chen, X.-R., Zhang, B.-Y., Xing, Y.-P., Li, Q.-Y., Li, Y.-P., Tong, Y.-H., and Xu, J.-Y. 2014. Transcriptomic analysis of the phytopathogenic oomycete *Phytophthora cactorum* provides insights into infection-related effectors. *BMC Genomics* 15:980.
- Conesa, A., Götz, S., García-Gómez, J. M., Terol, J., Talón, M., and Robles, M. 2005. Blast2GO: A universal tool for annotation, visualization and analysis in functional genomics research. *Bioinformatics* 21:3674-3676.
- Costanzo, S., Ospina-Giraldo, M. D., Deahl, K. L., Baker, C. J., and Jones, R. W. 2006. Gene duplication event in family 12 glycosyl hydrolase from *Phytophthora* spp. *Fungal Genet. Biol.* 43:707-714.
- Davidson, N. M., and Oshlack, A. 2014. Corset: Enabling differential gene expression analysis for de novo assembled transcriptomes. *Genome Biol.* 15:410.
- Davik, J., Eikemo, H., Brurberg, M. B., and Sargent, D. J. 2015. Mapping of the *RpC-1* locus for *Phytophthora cactorum* resistance in *Fragaria vesca*. *Mol. Breed.* 35:211.
- Denoyes-Rothan, B., Lerceteau-Köhler, E., Guérin, G., Bosseur, S., Bariac, J., Martin, E., and Roudeillac, P. 2004. QTL analysis for resistances to *Colletotrichum acutatum* and *Phytophthora cactorum* in octoploid strawberry (*Fragaria × ananassa*). *Acta Hort.* 663:147-152.
- Derevnina, L., Dagdas, Y. F., De la Concepcion, J. C., Bialas, A., Kellner, R., Petre, B., Domazakis, E., Du, J., Wu, C. H., Lin, X., and Aguilera-Galvez, C. 2016. Nine things to know about elicitors. *New Phytol.* 212:888-895.
- Dong, S., Kong, G., Qutob, D., Yu, X., Tang, J., Kang, J., Dai, T., Wang, H., Gijzen, M., and Wang, Y. 2012. The NLP toxin family in *Phytophthora sojae* includes rapidly evolving groups that lack necrosis-inducing activity. *Mol. Plant-Microbe Interact.* 25:896-909.
- Eikemo, H., Klemsdal, S. S., Riisberg, I., Bonants, P., Stensvand, A., and Tronsmo, A. M. 2004. Genetic variation between *Phytophthora cactorum* isolates differing in their ability to cause crown rot in strawberry. *Mycol. Res.* 108:317-324.
- Erwin, D. C., and Ribeiro, O. K. 1996. *Phytophthora* Diseases Worldwide. American Phytopathological Society, St. Paul, MN.

- Finn, R. D., Clements, J., Arndt, W., Miller, B. L., Wheeler, T. J., Schreiber, F., Bateman, A., and Eddy, S. R. 2015. HMMER web server: 2015 update. *Nucleic Acids Res.* W30-W38.
- Grenville-Briggs, L. J., Kushwaha, S. K., Cleary, M. R., Witzell, J., Savenkov, E. I., Whisson, S. C., Chawade, A., and Vetukuri, R. R. 2017. Draft genome of the oomycete pathogen *Phytophthora cactorum* strain LV007 isolated from European beech (*Fagus sylvatica*). *Genom. Data* 12:155-156.
- Haas, B. J., Kamoun, S., Zody, M. C., Jiang, R. H., Handsaker, R. E., Cano, L. M., Grabherr, M., Kodira, C. D., Raffaele, S., Torto-Alalibo, T., Bozkurt, T. O., Ah-Fong, A. M., Alvarado, L., Anderson, V. L., Armstrong, M. R., Avrova, A., Baxter, L., Beynon, J., Boevink, P. C., Bollmann, S. R., Bos, J. I., Bulone, V., Cai, G., Cakir, C., Carrington, J. C., Chawner, M., Conti, L., Costanzo, S., Ewan, R., Fahlgren, N., Fischbach, M. A., Fugelstad, J., Gilroy, E. M., Gnerre, S., Green, P. J., Grenville-Briggs, L. J., Griffith, J., Grünwald, N. J., Horn, K., Horner, N. R., Hu, C. H., Huitema, E., Jeong, D. H., Jones, A. M., Jones, J. D., Jones, R. W., Karlsson, E. K., Kunjeti, S. G., Lamour, K., Liu, Z., Ma, L., Maclean, D., Chibucos, M. C., McDonald, H., McWalters, J., Meijer, H. J., Morgan, W., Morris, P. F., Munro, C. A., O'Neill, K., Ospina-Giraldo, M., Pinz'ón, A., Pritchard, L., Ramsahoye, B., Ren, Q., Restrepo, S., Roy, S., Sadanandom, A., Savidor, A., Schornack, S., Schwartz, D. C., Schumann, U. D., Schwessinger, B., Seyer, L., Sharpe, T., Silvar, C., Song, J., Studholme, D. J., Sykes, S., Thines, M., van de Vondervoort, P. J., Phuntumart, V., Wawra, S., Weide, R., Win, J., Young, C., Zhou, S., Fry, W., Meyers, B. C., van West, P., Ristaino, J., Govers, F., Birch, P. R., Whisson, S. C., Judelson, H. S., and Nusbaum, C. 2009. Genome sequence and analysis of the Irish potato famine pathogen *Phytophthora infestans*. *Nature* 461:393-398.
- Haas, B. J., Papanicolaou, A., Yassour, M., Grabherr, M., Blood, P. D., Bowden, J., Couger, M. B., Eccles, D., Li, B., Lieber, M., Macmanes, M. D., Ott, M., Orvis, J., Pochet, N., Strozzi, F., Weeks, N., Westerman, R., Williams, T., Dewey, C. N., Henschel, R., Leduc, R. D., Friedman, N., and Regev, A. 2013. *De novo* transcript sequence reconstruction from RNA-seq using the Trinity platform for reference generation and analysis. *Nat. Protoc.* 8:1494-1512.
- Hamm, P. B., and Hansen, E. M. 1991. The isolation and identification of *Phytophthora* species causing damage in bare-root conifer nurseries. Pages 169-179 in: *Proceedings of the First Meeting of the IUFO Working Party S2.07-09 (Diseases and Insects in Forest Nurseries)*. J. R. Sutherland and S. G. Glover, eds. Forestry Canada, Victoria, BC, Canada.
- Hantula, J., Lilja, A., Nuorteva, H., Parikka, P., and Werres, S. 2000. Pathogenicity, morphology and genetic variation of *Phytophthora cactorum* from strawberry, apple, rhododendron, and silver birch. *Mycol. Res.* 104:1062-1068.
- Huang, H., Jeffers, S. N., Layne, D. R., and Schnabel, G. 2004. AFLP analysis of *Phytophthora cactorum* isolates from strawberry and other hosts: Implications for identifying the primary source of inoculum. *Plant Dis.* 88: 714-720.
- Ivanov, D. A., and Bernard, M. A. 2012. Ginsenosidases and the pathogenicity of *Pythium irregulare*. *Phytochemistry* 78:44-53.
- Jia, Y. J., Feng, B. Z., Sun, W. X., and Zhang, X. G. 2009. Polygalacturonase, pectate lyase and pectin methylesterase activity in pathogenic strains of *Phytophthora capsici* incubated under different conditions. *J. Phytopathol.* 157:585-591.
- Jiang, R. H., Tyler, B. M., Whisson, S. C., Hardham, A. R., and Govers, F. 2006. Ancient origin of elicitor gene clusters in *Phytophthora* genomes. *Mol. Biol. Evol.* 23:338-351.
- Jones, P., Binns, D., Chang, H., Fraser, M., Li, W., McAnulla, C., McWilliam, H., Maslen, J., Mitchell, A., and Nuka, G. 2014. InterProScan 5: Genome-scale protein function classification. *Bioinformatics* 30:1236-1240.
- Kamoun, S., Young, M., Förster, H., Coffey, M. D., and Tyler, B. M. 1994. Potential role of elicitors in the interaction between *Phytophthora* species and tobacco. *Appl. Environ. Microbiol.* 60:1593-1598.
- Koskinen, P., Törönen, P., Nokso-Koivisto, J., and Holm, L. 2015. PANNZER: High-throughput functional annotation of uncharacterized proteins in an error-prone environment. *Bioinformatics* 31:1544-1552.
- Langmead, B., Trapnell, C., Pop, M., and Salzberg, S. L. 2009. Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. *Genome Biol.* 10:R25.
- Lee, H.-A., Kim, S.-Y., Oh, S.-K., Yeom, S.-I., Kim, S.-B., Kim, M.-S., Kamoun, S., and Choi, D. 2014. Multiple recognition of RXLR effectors is associated with nonhost resistance of pepper against *Phytophthora infestans*. *New Phytol.* 203:926-938.
- Livak, K. J., and Schmittgen, T. D. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2- $\Delta\Delta C_T$ method. *Methods* 25:402-408.
- Maas, J. L. 1998. Compendium of Strawberry Diseases. American Phytopathological Society, St. Paul, MN, St. Paul, MN.
- Maere, S., Heymans, K., and Kuiper, M. 2005. BiNGO: A Cytoscape plugin to assess overrepresentation of gene ontology categories in biological networks. *Bioinformatics* 21:3448-3449.
- Mangandi, J., Verma, S., Osorio, L., Peres, N. A., van de Weg, E., and Whitaker, V. M. 2017. Pedigree-based analysis in a multiparental population of octoploid strawberry reveals QTL alleles conferring resistance to *Phytophthora cactorum*. *G3 (Bethesda)* 7:1707-1719.
- Manter, D. K., Kolodny, E. H., Hansen, E. M., and Parke, J. L. 2010. Virulence, sporulation, and elicitor production in three clonal lineages of *Phytophthora ramorum*. *Physiol. Mol. Plant Pathol.* 74:317-322.
- Martinez, S. E., Beavo, J. A., and Hol, W. G. 2002. GAF domains: Two-billion-year-old molecular switches that bind cyclic nucleotides. *Mol. Interv.* 2:317-323.
- Na, R., and Gijzen, M. 2016. Escaping host immunity: New tricks for plant pathogens. *PLoS Pathog* 12:e1005631.
- Neculai, M. A., Ivanov, D., and Bernard, M. A. 2009. Partial purification and characterization of three ginsenoside-metabolizing β -glucosidases from *Pythium irregulare*. *Phytochemistry* 70:1948-1957.
- Nellist, C. F., Vickerstaff, R., Sobczyk, M. K., Marina-Montes, C., Brain, P., Wilson, F. M., Simpson, D. W., Whitehouse, A. B., and Harrison, R. 2018. Quantitative trait loci controlling *Phytophthora cactorum* resistance in the cultivated octoploid strawberry (*Fragaria × ananassa*). *bioRxiv* 249573.
- Ochiai, N., Dragila, M. I., and Parke, J. L. 2011. Pattern swimming of *Phytophthora citricola* zoospores: An example of microbial bioconvection. *Fungal Biol.* 115:228-235.
- Pais, M., Yoshida, K., Giannakopoulou, A., Pel, M. A., Cano, L. M., Oliva, R. F., Witek, K., Lindqvist-Kreuz, H., Vleeshouwers, V. G. A. A., and Kamoun, S. 2018. Gene expression polymorphism underpins evasion of host immunity in an asexual lineage of the Irish potato famine pathogen. *BMC Evol. Biol.* 18:93.
- Robinson, M. D., McCarthy, D. J., and Smyth, G. K. 2010. edgeR: A Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics* 26:139-140.
- Rytkönen, A., Lilja, A., Vercauteren, A., Sirkkä, S., Parikka, P., Soukainen, M., and Hantula, J. 2012. Identity and potential pathogenicity of *Phytophthora* species found on symptomatic *Rhododendron* plants in a Finnish nursery. *Can. J. Plant Pathol.* 34:255-267.
- Seidl, M. F., Van den Ackerveken, G., Govers, F., and Snel, B. 2011. A domain-centric analysis of oomycete plant pathogen genomes reveals unique protein organization. *Plant Physiol.* 155:628-644.
- Shaw, D. V., Hansen, J., and Browne, G. T. 2006. Genotypic variation for resistance to *Phytophthora cactorum* in a California strawberry breeding population. *J. Am. Soc. Hortic. Sci.* 131:687-690.
- Shaw, D. V., Hansen, J., Browne, G. T., and Shaw, S. M. 2008. Components of genetic variation for resistance of strawberry to *Phytophthora cactorum* estimated using segregating seedling populations and their parent genotypes. *Plant Pathol.* 57:210-215.
- Shulaev, V., Sargent, D. J., Crowhurst, R. N., Mockler, T. C., Folkerts, O., Delcher, A. L., Jaiswal, P., Mockaitis, K., Liston, A., Mane, S. P., Burns, P., Davis, T. M., Slovin, J. P., Bassil, N., Hellens, R. P., Evans, C., Harkins, T., Kodira, C., Desany, B., Crasta, O. R., Jensen, R. V., Allan, A. C., Michael, T. P., Setubal, J. C., Celton, J.-M., Rees, D. J. G., Williams, K. P., Holt, S. H., Rojas, J. J. R., Chatterjee, M., Liu, B., Silva, H., Meisel, L., Adato, A., Filichkin, S. A., Troglio, M., Viola, R., Ashman, T.-L., Wang, H., Dharmawardhana, P., Elser, J., Raja, R., Priest, H. D., Bryant, D. W., Jr., Fox, S. E., Givan, S. A., Wilhelm, L. J., Naithani, S., Christoffels, A., Salama, D. Y., Carter, J., Girona, E. L., Zedepski, A., Wang, W., Kerstetter, R. A., Schwab, W., Korb, S. S., Davik, J., Monfort, A., Denoyes-Rothan, B., Arus, P., Mittler, R., Flinn, B., Aharoni, A., Bennetzen, J. L., Salzberg, S. L., Dickerman, A. W., Velasco, R., Borodovsky, M., Veilleux, R. E., and Folta, K. M. 2011. The genome of woodland strawberry (*Fragaria vesca*). *Nat. Genet.* 43:109-116.
- Simão, F. A., Waterhouse, R. M., Ioannidis, P., Kriventseva, E. V., and Zdobnov, E. M. 2015. BUSCO: Assessing genome assembly and annotation completeness with single-copy orthologs. *Bioinformatics* 31: 3210-3212.
- Sperschneider, J., Catanzariti, A., DeBoer, K., Petre, B., Gardiner, D., and Singh, K. 2017. LOCALIZER: Subcellular localization prediction of plant and effector proteins in the plant cell. *Sci. Rep.* 7:44598.
- Toljamo, A., Blande, D., Kärenlampi, S., and Kokko, H. 2016. Reprogramming of Strawberry (*Fragaria vesca*) root transcriptome in response to *Phytophthora cactorum*. *PLoS One* 11:e0161078.
- Thompson, J. R., Wetzel, S., Klerks, M. M., Vašková, D., Schoen, C. D., Špak, J., and Jelkmann, W. 2003. Multiplex RT-PCR detection of four aphid-borne strawberry viruses in *Fragaria* spp. in combination with a plant mRNA specific internal control. *J. Virol. Methods* 111:85-93.
- Toljamo, A. R., Granlund, L. J., and Kokko, H. I. 2017. DNA barcode identification and virulence testing of *Phytophthora* spp. and *Pythium* spp. isolated from soil of strawberry fields. *Acta Hortic.* 727-734.
- Unestam, T. 1965. Studies on the crayfish plague fungus *Aphanomyces astaci* I. Some factors affecting growth in vitro. *Physiol. Plant.* 18: 483-505.

- Untergasser, A., Cutcutache, I., Koressaar, T., Ye, J., Faircloth, B. C., Remm, M., and Rozen, S. G. 2012. Primer3—new capabilities and interfaces. *Nucleic Acids Res.* 40:e115.
- Van Der Scheer, H. A. Th. 1971. Isolation of *Phytophthora cactorum* from soil in orchards and strawberry fields and differences in pathogenicity to apple. *Neth. J. Plant Pathol.* 77:65-72.
- VanEtten, H., Temporini, E., and Wasmann, C. 2001. Phytoalexin (and phytoanticipin) tolerance as a virulence trait: Why is it not required by all pathogens? *Physiol. Mol. Plant Pathol.* 59:83-93.
- Vauthrin, S., Mikes, V., Milat, M., Ponchet, M., Maume, B., and Osman, H. 1999. Elicitins trap and transfer sterols from micelles, liposomes and plant plasma membranes. *Biochim. Biophys. Acta.* 1419:335-342.
- Vega-Arreguín, J. C., Jalloh, A., Bos, J. I., and Moffett, P. 2014. Recognition of an Avr3a homologue plays a major role in mediating nonhost resistance to *Phytophthora capsici* in *Nicotiana* species. *Mol. Plant-Microbe Interact.* 27:770-780.
- Wang, Q., Han, C., Ferreira, A. O., Yu, X., Ye, W., Tripathy, S., Kale, S. D., Gu, B., Sheng, Y., Sui, Y., Wang, X., Zhang, Z., Cheng, B., Dong, S., Shan, W., Zheng, X., Dou, D., Tyler, B. M., and Wang, Y. 2011. Transcriptional programming and functional interactions within the *Phytophthora sojae* RXLR effector repertoire. *Plant Cell* 23:2064-2086.
- Yan, H., and Liou, R. 2006. Selection of internal control genes for real-time quantitative RT-PCR assays in the oomycete plant pathogen *Phytophthora parasitica*. *Fungal Genet. Biol.* 43:430-438.
- Yang, M., Duan, S., Mei, X., Huang, H., Chen, W., Liu, Y., Guo, C., Yang, T., Wei, W., Liu, X., He, X., Dong, Y., and Shusheng, Z. 2018. The *Phytophthora cactorum* genome provides insights into the adaptation to host defense compounds and fungicides. *Sci. Rep.* 8:6534.
- Yin, Y., Mao, X., Yang, J., Chen, X., Mao, F., and Xu, Y. 2012. dbCAN: A web resource for automated carbohydrate-active enzyme annotation. *Nucleic Acids Res.:* W445-W451.
- Zentmyer, G. A., and Chen, D. W. 1969. Production of sporangia by *Phytophthora cinnamomi* in pure culture. *Calif. Avocado Soc. Yearb.* 53:103-107.
- Zhang, H., Yohe, T., Huang, L., Entwistle, S., Wu, P., Yang, Z., Busk, P. K., Xu, Y., and Yin, Y. 2018. dbCAN2: a meta server for automated carbohydrate-active enzyme annotation. *Nucleic Acids Res.* 46:W95-W101.