1 Identification of Pathogenicity-Related Genes and the Role of a 2 Subtilisin-Related Peptidase S8 (PePRT) in Authophagy and 3 Virulence of *Penicillium expansum* on Apples

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Abstract

Blue mold caused by *Penicillium expansum* is a major postharvest disease of pome fruit. Several mechanisms possibly involved in *P. expansum* pathogenicity and virulence. However, factors that mediate pathogenicity and virulence are largely not yet characterized. In this work we analyzed *P. expansum* predicted secretome to reveal potential genes that have a role in host-pathogen interaction. A prediction pipeline was designed using an approach that combines common effector features, transcriptomic data and homology to proteins reported to be involved in pathogenicity of other phytopathogenic fungi. Among 297 genes predicted in *P. expansum* secretome, 103 genes (35 %) were found to code for hydrolytic enzymes. The majority of the secreted enzymes are carbohydrate-degrading enzymes among which five coding for pectin-degrading enzymes are highly induced during the infection and decay of apple fruit by *P. expansum*, indicating that they may represent an important aspect of pathogenicity and virulence. Applying the pipeline we have predicted 17 candidate genes coding for proteins that are most likely involved in pathogenicity and virulence. One of the top candidates is a subtilisin-related peptidase, S8 (PePRT), proteolytic enzyme highly expressed *in planta*, and potentially involved in autophagy process. Deletion of PePRT-coding gene resulted in reduced virulence of *P. expansum* on apples. Moreover, Δ Peprt exhibited decreased sporulation as well as affected mycelial morphology and internal mycelial cell structure.

1. Introduction

Penicillium is a fungal genus with 354 currently-recognized species, distributed worldwide on various substrates ranging from soil to food. *P. expansum* Link, (1809) is a necrotrophic pathogen infecting a wide range of fruits, including pome and various stone fruit (Cappellini et al. 1987,

40 Jurick et al. 2011). Necrotrophs cause host cell death in order to utilize nutrients from the dead
41 tissue for proliferation and growth. There is growing recognition of the complexity of the
42 interaction between necrotrophic fungi and their hosts, which involves sophisticated recognition
43 and signaling networks. In plants, there are two known layers of immune systems. First layer is
44 PTI (pathogen-associated molecular pattern (PAMP)-triggered immunity). Conserved pathogen
45 molecules are recognized by pattern recognition receptors, which triggers wide-ranging immune
46 response. The second layer is elicited as a result of recognition of variable pathogen effectors by
47 host disease-resistance proteins. This type of recognition induces a rapid and robust hypersensitive
48 response (HR) (Boller and He 2009). A wide-range of virulence strategies are used by necrotrophic
49 fungi to overcome host innate immune systems, including the production of diverse phytotoxic
50 compounds, cell wall-degrading enzymes and proteinaceous effectors (Wang et al. 2014). The
51 necrotrophic pathogens *Stagonospora nodorum* and *Pyrenophoratrutici-repentis* produce multiple
52 proteinaceous host-selective toxins that function in effector-triggered susceptibility (Abeysekara
53 et al. 2009, Friesen et al. 2007, Friesen et al. 2008, Liu et al. 2009, Liu et al. 2012, Manning et al.
54 2007). Necrosis and ethylene-inducing peptide 1 (NEP1)-like (NLP) effectors, produced by many
55 plant pathogens, are also found in necrotrophic fungi (Dallal Bashi et al. 2010, Schouten et al.
56 2008) . The role of LysM effectors in pathogenicity and virulence in several pathogen-plant
57 systems has also been reported (Bolton et al. 2008, de Jonge et al. 2010). We previously
58 characterized the role of four LysM proteins in the virulence of *P. expansum* on apple fruit.
59 Although single knockouts of each of the four genes had no significant effect on pathogenicity and
60 virulence, yeast two-hybrid (Y2H) study using the LysM domain as a bait, detected broad-
61 spectrum of interacting apple proteins, including proteins involved in plant defense (Levin et al.
62 2017). These findings suggest the possibility that *P. expansum* LysM proteins may play a role in

modulating host cellular processes and the subsequent development of decay. The identification and functional role of virulence proteins in the majority of necrotrophic pathogens, however, has not been accomplished.

Several studies have designed computational tools to predict effectors, now that there is an ever increasing number of sequenced genomes of plant pathogenic fungi (reviewed in (Sperschneider et al. 2015)). Fungal effector prediction is challenging due to the lack of common features in effectors within and across species. In general, parameters such as small size, presence of a secretion signal, and a large number of cysteine residues are used as criteria in fungal effector prediction. This approach, however, results in numerous false-positive and false-negative candidates. Recently, more sophisticated techniques for predicting apoplastic and cytoplasmic effector candidates have been reported (Sperschneider et al. 2013, Sperschneider et al. 2014, Syme et al. 2013). Guyon et al. (2014) identified a total of 486 secreted protein genes in *S. sclerotiorum* likely to be involved in the interaction between the fungus and its hosts. Furthermore, the following criteria were used to identify a total of 78 putative effectors: (i) protein domains and motifs found in known fungal effectors, (ii) signatures of positive selection, (iii) recent gene duplication, or (iv) *S. sclerotiorum*-specific genes (Guyon et al. 2014).

In parallel to the increase in the number of available fungal plant pathogen genome sequences, the body of experimental data, and the number of publications on pathogenicity and virulence associated genes are also constantly growing. The Pathogen Host Interaction database (PHI-base; <http://www.phi-base.org/index.jsp>) is a web-accessible database that catalogues experimentally verified pathogenicity- and virulence-related genes in fungal, oomycete, and bacterial pathogens in animal, plant, and insect hosts. Each entry in PHI-base is curated by domain experts and is supported by strong experimental evidence (Urban et al. 2016).

In the present study, we report on the identification of potential genes that are possibly involved in pathogenicity/virulence of *P. expansum* using an approach that combines common effector features with transcriptomic data and evidence of pathogenicity reported in the literature. A pipeline for prediction of pathogenicity-related genes in *P. expansum* was constructed and its use revealed the presence of 17 candidate genes. More specifically, a subtilisin-related peptidase, S8 (PePRT), was found to possess most prevalent features, as defined in our prediction pipeline. PePRT was subsequently characterized and functionally analyzed. Deletion of the *prt* gene in *P. expansum* affected virulence on apples, as well as mycelial morphology, cell structure and sporulation.

2. Experimental Procedures

2.1 Plant material and fungal cultures

P. expansum PEX2 (MD-8, Accession: PRJNA255747 ID:255747), not producing patulin strain isolated in USA from an infected apple fruit, described by Ballester et al (2015), was used throughout all experiments. Apple fruit (cv. Golden Delicious) were sampled at harvest maturity and used shortly after collection or stored at 0 °C until further processing. Fruit were disinfected prior to inoculation by dipping them in 0.05 % sodium hypochlorite for 2 min and followed by a thorough rinse with tap water.

P. expansum was cultivated on potato dextrose agar (PDA) or in potato dextrose broth (PDB) at 25 °C. Spore suspensions were prepared by harvesting the spores with a sterile bacteriological loop from 2 to 3-week-old PDA cultures and suspending them in sterile distilled water. The suspension was filtered through four layers of sterile cheesecloth to remove residual mycelium, and spores were washed twice with sterile distilled water by centrifugation at 8,000 x g for 5 min and

resuspension in sterile distilled water. Spore concentration was determined using hemocytometer and adjusted as needed.

2.2 Secretome prediction and annotation

A pipeline similar to that described by Mueller et al (2008) to predict the secretome of *Ustilago maydis* was used to predict the secretome of *P. expansum* with minor modifications (Mueller et al. 2008). Predicted protein set of PEX1 (*P. expansum* CMP-1, Accession: PRJNA255744 ID:255744) and PEX2 were downloaded from the National Center for Biotechnology Information (NCBI) database under the accession numbers JQFX01000000 and JQFZ01000000, respectively (Ballester et al. 2015). *In silico* prediction of extracellular proteins was performed using SignalP (v4.1; <http://www.cbs.dtu.dk/services/SignalP/>) to determine the presence of signal peptides (SSPs) (Nielsen 2017, Petersen et al. 2011). The TargetP server (v1.1; <http://www.cbs.dtu.dk/services/TargetP/>) was used to identify and remove SSPs with either a chloroplast transit peptide or a mitochondrial targeting peptide (Loc=S and Reliability Class ≤ 2) (Emanuelsson et al. 2007, Petersen et al. 2011). TMHMM software (v2.0; <http://www.cbs.dtu.dk/services/TMHMM/>) was then utilized to predict and remove transmembrane (TM) helices containing proteins, except those with one TM in the N-terminal signal peptide (Krogh et al. 2001). The remaining SSPs were screened for the presence of glycosylphosphatidylinositol (GPI) anchor proteins using big-PI (http://mendel.imp.ac.at/gpi/fungi_server.html) (Eisenhaber et al. 2004). Finally, the selected SSPs were subjected to PFAM analysis using the PFAM database (v27.0; <http://pfam.xfam.org/search#tabview=tab1>) (Finn et al. 2016) and to InterProScan (v5.1; <http://www.ebi.ac.uk/interpro/>) (Jones et al. 2014). Evidence for *in planta* expression was

obtained based on the results of RNASeq analysis of four different libraries: a cDNA library synthesized from a mixture of RNAs obtained from *P. expansum* PEX1 spores collected after 7 d of growth on PDA, healthy non-inoculated fruit, and three libraries from *P. expansum*-infected apples at 24, 48, and 72 h post-inoculation (hpi), respectively (Ballester et al. 2015). The raw RNA-Seq data files were downloaded from the NCBI database under SRA accession number SRP043407.

2.3 Pathogenicity-related factor prediction pipeline

Based on the results of secretome prediction and annotation, five groups of potential pathogenicity-related factors were selected: 1) small, cysteine-rich - non-annotated proteins smaller than 300 amino acids with 4 or more cysteines; 2) proteins highly induced *in planta*: genes whose expression level, based on the RNAseq data, was equal to or greater than an average expression level plus two standard errors; 3) proteins with a nuclear localization signal (NLS), predicted using NLStradamus software (Revision r.9) (Nguyen Ba et al. 2009); 4) proteins that belong to known effector families or carrying effector-domains previously reported in the literature; and 5) proteins homologous to gene-products proven to affect the outcome of pathogen-host interactions predicted by BlastP homology analysis against PHI-base v.3.8 (Urban et al. 2016). Gene products fulfilling two or more of these five characteristics were selected as the “top candidates”.

2.4 Fruit inoculation and disease assessment

Apple fruit at harvest maturity were wounded at four to six different sites around the equator to a depth of 4 mm using a 1.25-mm diameter needle. Then, 10 µl of a spore suspension of *P. expansum* (10^6 spores mL⁻¹) was pipetted into each wound. A range of 8-12 fruit were used and each

experiment was repeated three times. Inoculated fruit were placed in plastic trays lined with filter paper soaked with sterile water (150 mL per tray) and incubated in darkness at 25°C. Percent infection and average rot diameter were determined starting from the 3rd day after inoculation.

2.5 RNA extraction from *P. expansum*-infected apple tissue and cDNA synthesis

Total RNA was extracted from *P. expansum*-infected apple fruit tissue at different times starting 24 h post inoculation (hpi). That is based on the previous work in which the transcriptome of *P. expansum* during apple infection was characterized (Ballester et al. 2015). There were three biological replicated and each replicate consisted of decayed tissue collected from 5 fruit (4 infected sites/fruit). RNA extraction was done following the protocol described by Levin et al. (2017). Synthesis of the first- cDNA strand was done using 1 µg of total RNA that had been pretreated with 0.25 U of recombinant DNase I (Takara Bio Inc., Shiga, Japan), using the Verso cDNA Kit (Thermo Fisher Scientific, Epsom, UK) following manufacturer's guidelines.

2.6 PCR and Reverse transcription – quantitative PCR (RT-qPCR)

PCR reactions were performed using standard thermocycler (Senso Quest, Göttingen, Germany). Each 10 µL of PCR reaction contained 1U DreamTaq DNA polymerase (Thermo Scientific), DreamTaq buffer, 0.2 mM dNTPs, 0.4 µM of each primer and 1 ng–1 µg template DNA. PCR conditions were: 5 min at 94 °C, followed by 40 cycles of 30 s at 94 °C, 15 s at 54-58 °C, 1-3 min at 72 °C; and 10 min at 72 °C. Primer sequences are listed in Table S1.

RT-qPCR was performed with a StepOnePlus real time PCR system (Applied Biosystems, Foster City, United States). PCR amplification was performed with 4 µL of cDNA template in 10 µL of

a reaction mixture containing 5 μ L of Fast SYBR Green Master Mix (Applied Biosystems), and 300 nM primers (PepI_F/PepI_R for PePRT and 28S_F/28S_R for 28S rRNA, Table S1). Results were analyzed using StepOnePlus software. Gene expression was determined as a function of time, 28S rRNA served as an endogenous control. Samples collected at 0 h post-inoculation (hpi) were used to calibrate expression levels. A mixture of cDNAs from all the samples was used in the analysis of all the treatments as a template to generate calibration curves designed for each pair of primers. Relative quantification was calculated using the mathematical model of Pfaffl (2001) in which the relative expression ratio of a target gene is calculated based on the efficiency and the cross point deviation of an unknown sample versus a control, and expressed in comparison to the reference gene indicated above (Pfaffl 2001). A combination of three biological replicates with three technical replicates of each reaction were used.

2.7 Construction and analysis of *PePRT* knockout mutant

Knockout mutants were constructed following the protocol used by Ballester et al. (2015). Flanking regions of the 5' upstream (promoter) and 3' downstream (terminator) of *Peprt* gene (PEX2_027670) were PCR amplified from genomic DNA of *P. expansum* (Pe100/PEX2) using Prt_Prom_F/Prt_Prom_R and Prt_Ter_F/Prt_Ter_R primer pairs, respectively (Table S1). The PCR conditions were: 4 min at 94 °C, then 40 cycles of 30 s at 94 °C, 15 s at 56 °C, 2 min at 72 °C, and 10 min at 72 °C as final elongation step. DNA fragments corresponding to the promoter and terminator regions and the pRFHU2 vector (Frandsen et al. 2008) previously digested with *PacI* and *Nt.BbvCI* (New England Biolabs) were mixed together and then treated with USER enzyme (New England Biolabs) to obtain the pRFHU2-PePrt plasmid. An aliquot of the mixture was used directly for chemical transformation of *Escherichia coli* JM109 cells (Promega) without

prior ligation. Kanamycin-resistant transformants were screened by PCR for the presence of promoter and terminator with primer pairs RF-1/RF-6 and RF-2/RF-5, respectively (Table S1). Proper insertion was confirmed by DNA sequencing. The selected plasmids were introduced into chemically competent *Agrobacterium tumefaciens* AGL-1 cells, which were subsequently used to transform *P. expansum* PEX2, as described previously (Buron-Moles et al. 2012). Analysis of transformants for gene disruption events was done by PCR. The insertion of the selection marker was checked with primer pair HMBF1/HMBR1 (Table S1). Integration of the T-DNA by homologous recombination was examined using primer pairs HPH1F/Prt_center_F and HPHPRO4/Prt_center_R (Table S1). Further verification of deletion of the target gene was done with primer pairs Prt_center_F/Prt_center_R (Table S1). Real-time genomic PCR analysis was carried out in order to determine the number of T-DNA copies that had been integrated into the genome of the transformants, following the protocol described by Crespo-Sempere et al. (2013), using PEX2 as the control (Crespo-Sempere et al. 2013). Primer pair Prt_RT_F/Prt_RT_R was designed within the T-DNA in the promoter regions of PePrt, close to the selection marker. The *P. expansum* β -tubulin gene (AY674401) (Sanzani et al. 2009) was chosen as reference gene using the primer pair PeTub_1F/PeTub_2R.

2.8 Radial growth, sporulation, and spore germination assays

A droplet of 5 μ L of spore suspension at 10^6 spore mL^{-1} was placed in the center of PDA plates and incubated at 25 °C. Radial growth of the cultures was measured daily. 5 mL of distilled water were added to eleven days-old PDA plates, and the spores were collected using a Digiralsky stick. Each plate was washed with an additional 2 mL of distilled water to insure maximum spore collection. The resulting spore suspensions were diluted and spore concentrations were counted

with a hemocytometer. The number of spores per plate was calculated as follows: number of spores per plate = spore concentration (spores mL⁻¹) * dilution factor * 7mL.

In order to determine the percentage of germinating spores and germ-tube length, droplets of 15 µL of spores suspension at 10⁵ spores mL⁻¹ were placed at several locations on water-agar plates (1.5 % agar). The plates were observed under a microscope following incubation at 25 °C for 18 h, and image analysis was used to calculate percent germination and germ-tube length using NIS-Elements BR Microscope Imaging Software (Nikon Instruments Inc., USA).

2.9 Microscopy

2.9.1 Scanning electron microscopy:

Samples of mycelia were taken from ten-day-old colonies growing on PDA plates. Each sample was immobilized on a holder using carbon tape and image acquisition was done using a tabletop scanning electron microscope TM3030Plus (Hitachi).

2.9.2 Confocal microscopy:

P. expansum was grown on PDA for one week in a glass petri dish. Images were acquired with a Leica SP8 laser scanning microscope (Leica, Wetzlar, Germany), equipped with a solid state laser with 448 nm light and HCX PL APO CS 10x/0.40 dry or HC PL APO CS 60x/1.2 water immersion objectives (Leica, Wetzlar, Germany), and Leica Application Suite X software (LASX, Leica, Wetzlar, Germany). Vacuoles were stained by immersing and incubating mycelial samples in 5 mgr L⁻¹ FM4-64 (Molecular Probes) for 15 min at room temperature. FM4-64 emission signal was detected with HyD (hybrid) detector in the range of 580-680 nm.

3. Results and Discussion

246

247 3.1 Secretome analysis

248 The genome sequence and annotation of *P. expansum* strain PEX2 was previously described by
249 Ballester et al. (2015). Analysis of the predicted secretome in *P. expansum* strain PEX2, which
250 possesses a total of 11,060 predicted proteins, was conducted in the present study in order to
251 identify potential secreted effector proteins. SignalP v4.1 and TargetP v1.1 were collectively used
252 to predict the total secretome (Fig. 1A). A total of 688 genes encoding secreted proteins were
253 identified, representing 6.2 % of the *P. expansum* PEX2 proteome. This percentage is similar to
254 the 7.7 % predicted by Li et al (2015) for *P. expansum*, strain T01, and for *P. italicum* (7.1 %), and
255 *P. digitatum* (6.0 %) (Li et al. 2015). The predicted values are slightly lower than those reported
256 for two other plant necrotrophs, *Sclerotinia sclerotiorum* (9.8 %) and *B. cinerea* (10 %) (Heard et
257 al. 2015). An analysis of the predicted secreted proteins using TMHMM v2.0 software, indicated
258 that 617 did not possess a transmembrane domain (TM) or a single TM in or just beyond, the signal

Figure 1: Secretome prediction pipeline (A) and pathogenicity-related genes prediction pipeline (B) of *Penicillium expansum*. Painted rectangles indicate groups of potential pathogenicity-related genes. Venn diagram representation of genes shared between groups of potential pathogenicity-related genes and considered as top candidates (C). Pink – small cysteine-rich proteins with unknown function, purple – genes highly expressed during apple infection, green - proteins homologous to gene-products proven to affect the outcome of pathogen-host interactions predicted by BlastP homology analysis against PHI-base v.3.8, yellow - proteins that belong to known effector families or carrying effector-domains previously reported in the literature, brown - proteins with a nuclear localization signal.

peptide. Using big-PI fungal prediction software (Eisenhaber et al. 2004), 64 proteins were subsequently predicted to harbor a GPI-lipid anchor, which more likely indicates a surface protein rather than a secreted protein. Thus, these proteins were removed from the list of potential secreted proteins, reducing the number of predicted secreted proteins to 553.

The largest group of enzymes among the predicted secreted proteins are similar to enzymes involved in metabolism of carbohydrates (CAZymes, 37 % of total secreted proteins). Most of these enzymes are glycoside hydrolases (approximately half of the enzymes that are associated with carbohydrate metabolism). A total of 21 of the enzymes secreted by PEX2 are involved in the degradation of pectin, such as polysaccharide lyases (PL1, PL3, and PL4), polygalacturonases (GH28), rhamnosidases (GH78), and pectin esterases (CE8, and CE12). Lignocellulose is a rigid complex formed by cellulose, hemicellulose, and lignin, and is the most abundant plant compound on the planet. In the current study, 40 secreted proteins were identified that are categorized as cellulases, xylanases, and hemicellulases (families GH6, GH7, GH10, GH11, GH12, among others). Two putative cutinases (CE5), involved in the degradation of the plant cuticle, were also present among the designated secreted proteins.

3.2 Prediction of genes potentially associated with pathogenicity.

Most bacterial and fungal pathogen effectors are considered to be species- or lineage-specific. This premise makes their prediction using universal features and/or functions problematic. Thus far, features such as i) secretion, ii) differential expression *in planta* during infection, iii) small size, and iv) large number of cysteines have been used in different computational analyses to identify putative effectors in fungal genomes (Mirzadi Gohari et al. 2015, Sonah et al. 2016, Sperschneider et al. 2015). In the current study, these four features, as well as information available in the PHI-

database on known pathogenicity/virulence related genes, were used to increase the likelihood of identifying genes relevant to processes associated with the infection of apples by *P. expansum*. Evidence of *in planta* expression was based on RNA-seq data reported by Ballester et al. (2015) for *P. expansum* PEX1. Protein identity between *P. expansum* PEX2 and PEX1 (isolated from decayed apples in Spain) is 99.7 %, (Ballester et al. 2015), indicating a high similarity between the two strains. Using the expression criterion the number of secretome-related genes was reduced from 553 to 297. Thus, the remaining predicted secretome contains genes that are actively transcribed during *P. expansum* infection and rot development in apples. Following the identification of putative secretome-related genes, the following criteria were employed to identify putative pathogenicity factors in the *P. expansum* secretome: 1) small (<300 aa) and cysteine-rich (>4) proteins with unknown function; 2) proteins with homology to known effectors and proteins containing PFAM domains found in fungal effectors described in the literature; 3) proteins with a nuclear localization signal (NLS), and 4) proteins that were homologous to gene-products proven to affect the outcome of pathogen-host interactions identified using BlastP against the PHI database. Concomitantly, to assist in selecting the best candidates, the expression levels of genes were examined, in order to identify those that were highly induced *in planta* (expression level equal to or larger than an average expression level plus two standard errors) (Fig. 1 B). Gene-products having at least two of these characteristics were categorized as top-candidates (Table 1). Lastly, genes coding cell-wall degrading enzymes were also identified and selected. The potential contribution of these latter genes to virulence is further discussed below.

304 **Table 1: Pathogenicity factor top-candidates.**

Gene name	size	# Cys	annotation	NLS ¹	PHI ²	High expression (hpi)
PEX2_002470	273	5				48, 72
PEX2_017860	1138	13	Pkinase; Ribonuclease_2-5A	+	1225; <i>G. zeae</i>	
PEX2_027670	496	3	Inhibitor_I9; Peptidase_S8	+	2117; <i>M. oryzae</i>	48, 72
PEX2_031340	378	8	Glyco-hyd 28		1027; <i>B. cinerea</i>	48, 72
PEX2_038290	138	10				48, 72
PEX2_047400	492	6	Thioredoxin	+	2644; <i>S. enterica</i>	
PEX2_048400	529	14	Glyco-hyd 72; X8		2808; <i>F. oxysporum</i>	48, 72
PEX2_056460	176	4				spores
PEX2_056540	515	9	Zinc finger, RING-type	+	2401; <i>X. campestris</i>	24
PEX2_065330	152	4	Cerato-platanin		3164; <i>T. virens</i>	
PEX2_076710	989	2	HSP70	+	2058; <i>M. oryzae</i>	
PEX2_080220	240	5	NPP1		2342; <i>B. elliptica</i>	24, 48, 72
PEX2_084770	259	1	TRAP_alpha (translokons associated protein)	+		24
PEX2_092040	297	4				24
PEX2_105000	289	9	WSC			72
PEX2_106730	1492	9	DNA glycosylase	+	2411; <i>S. sclerotiorum</i>	
PEX2_107230	377	6	Pectin lyase		3226; <i>P. digitatum</i>	24, 48

306 *Small, cysteine-rich secreted proteins*

307 Based on this approach, a total of 25 small, cysteine-rich secreted proteins with unknown function
308 were identified. Four of them (PEX2_002470, PEX2_038290, PEX2_056460, PEX2_092040)
309 exhibited a high level of expression *in planta*. PEX2_056460 was induced during spore
310 germination and the others during apple infection (Table S2).

311 *Proteins with homology to known effectors and proteins containing domains found in fungal*
312 *effectors*

313 An additional fifteen candidates were selected based on the presence of specific domains (Table
314 S3). PEX2_012920 contains a chitin binding domain (PF03067), PEX2_053590, PEX2_091440,
315 and PEX2_064530 have a LysM domain (PF01476; IPR018392), PEX2_002570 and
316 PEX2_105000 possess a WSC domain (PF01822.14), and PEX2_046410 has a carbohydrate
317 binding domain (PF02018.12). Another two proteins (PEX2_021580, PEX2_041810) possess a L-
318 like lectin domain (PF03388.8), and PEX2_028650 is annotated as a concanavalin A-like
319 lectin/glucanase containing a peptidase A4 domain (PF01828.12). Two proteins (PEX2_043920,
320 PEX2_092030) were found to have a cysteine-rich fungal effector motif (CEFM, PF05730), one
321 protein (PEX2_027670) contained a peptidase inhibitor I-9 domain (PF05922.11) and a subtilisin
322 (peptidase_ S8) domain (PF0082.17), one (PEX2_065330) is a ceratoplatanin (PF07249.7), and
323 one (PEX2_080220) is an NPP1-like (PF05630.6) protein. Only three of the designated candidates
324 were highly induced during apple infection: PEX2_105000 (WSC-containing domain),
325 PEX2_080220 (NPP1-like), and PEX2_027670 (a subtilase (peptidase S8) containing a protease
326 inhibitor domain). PEX2_027670 also has a NLS domain (Table S4) and is homologous to SPM1,

which has been reported to play an important role in the pathogenicity of *Magnaporthe oryzae* (Saitoh et al. 2009).

Proteins carrying NLS

Nineteen genes with a putative NLS were identified within the predicted secretome of *P. expansum* (Table S4). The occurrence of both a secretion signal and a NLS in the encoded proteins suggests that they may have a functional role in the plant nucleus. Three of the NLS-carrying proteins are highly expressed *in planta*: PEX2_027670 was mentioned above, PEX2_056540 encodes a zinc finger DNA binding protein homologous to CaRING1 from pepper, which is involved in the induction of cell death and the regulation of ubiquitination during the defense response to microbial pathogens (Lee et al. 2011), and PEX2_084770, a translocon-associated protein. Additional four NLS-carrying genes (PEX2_017860, PEX2_076710, PEX2_106730, PEX2_047400) were found to be homologous to genes reported to be involved in the pathogenicity of *Fusarium graminearum*, *M. oryzae*, *S. sclerotiorum*, and *Salmonella enterica*, respectively (Li et al. 2012, Sem and Rhen 2012, Wang et al. 2011, Yi et al. 2009). Interestingly, none of these proteins was shown to be functionally active in the host nucleus. PEX2_017860 is homologous to one of the 20 essential protein kinases in *F. graminearum* (Wang et al. 2011) while PEX2_106730 is homologous to a SS-Ggt1 γ -glutamyltranspeptidase in *S. sclerotiorum* that was shown to be involved in glutathione recycling during key developmental stages, such as the production of apothecia and appressoria (Li et al. 2012). PEX2_047400 is homologous to Thioredoxin-1 in *S. enterica*, which was shown to enhance virulence via the generation of systemic oxidative stress in the host (Sem and Rhen 2012). PEX2_076710 is a member of the heat shock protein 70 family and is homologous to the endoplasmic reticulum (ER) chaperone LHS1 in *M. oryzae*, and its deletion resulted in mutants that were severely impaired in penetration and biotrophic invasion of

susceptible rice (*Oryza sativa*) plants (Yi et al. 2009). LHS1 is not an effector by definition since it is localized in the ER, however, it is responsible for the proper processing of secreted effector proteins.

Proteins homologous to gene-products proven to affect the outcome of pathogen-host interactions

Overall, our bioinformatic approach identified 47 gene products that could potentially play a role in the pathogenicity of *P. expansum* on apple fruit based on homology search against PHI base (Table S5). The list included genes coding for carbohydrate-degrading enzymes, peptidases and lipases, redox proteins, proteins with oxidoreductase activity, chaperones, and proteins involved in signaling, DNA modifications and polyketide synthesis. In addition to the genes already mentioned (genes with a NLS, and those with a known effector-domain), the list contains three genes that exhibited a strong induction during apple infection: PEX2_048400 is a homologue of *gas-1* encoding a putative β -1,3glucanosyltransferase in *F. oxysporum* where it plays an active role in fungal cell wall biosynthesis and morphogenesis (Caracuel et al. 2005). Two additional genes, PEX2_031340 and PEX2_107230, are homologs of genes coding for pectin-degrading enzymes, *Bcpg1* (*Botrytis cinerea* endopolygalacturonase) (Have et al. 1998) and *pnl1* from *Penicillium digitatum* (López-Pérez et al. 2015).

Degrading enzymes

The analysis of the annotated genes in the *P. expansum* secretome revealed 103 genes (35 % of the total predicted in planta secretome) that code for hydrolytic enzymes (Table S6). The majority (73) of the secreted enzymes are carbohydrate-degrading enzymes that enable *P. expansum* to breakdown the complex carbohydrates in fruit tissue and use them as a source of nutrients. Some of the enzymes, such as the xylanases and pectin-degrading enzymes have been shown to be

372 important virulence factors in *Verticillium fungicola* (Amey et al. 2003), *M. oryzae* (Nguyen et al.
373 2011), *B. cinerea* (Akagi and Stotz 2007, Brito et al. 2006, Have et al. 1998, Valette-Collet et al.
374 2003), *Colletotrichum gloeosporioides* (Yakoby et al. 2001), and *P. digitatum* (López-Pérez et al.
375 2015). Five of the genes coding for pectin-degrading enzymes (PEX2_031340, PEX2_094840,
376 PEX2_110470, PEX2_107230, PEX2_092020) are highly induced during the infection and decay
377 of apple fruit by *P. expansum*, indicating that they may represent an important aspect of
378 pathogenicity and virulence.

379 Interestingly, another highly expressed gene, PEX2_048400, coding for a transglycosylase with a
380 cysteine-rich, carbohydrate-binding domain, was considered as putative pathogenicity factor in the
381 predicted secretome. Members of this family are usually GPI-anchored plasma membrane
382 enzymes that elongate and remodel the β -1,3 glucan of cell walls, thus playing an active role in
383 fungal cell wall biosynthesis and morphogenesis. An essential role for this type of enzyme during
384 the infection of tomato by *F. oxysporum* was reported by Caracuel et al. (2005). *F. oxysporum*
385 mutants lacking a functional copy of *gas1* exhibited significantly reduced levels of virulence on
386 tomato plants, thus providing the first evidence for an essential role of β -1,3-glucanosyltransferase
387 in the fungal pathogenesis of plants (Caracuel et al. 2005). Further analysis of catabolic enzymes
388 in the *P. expansum* secretome revealed 18 peptidases, one cutinase, one lipase, one
389 lysophospholipase, four enzymes that break down aromatic compounds, and four catabolic
390 enzymes that do not have a specific substrate (Table. S6).

391 Our analysis revealed four proteolytic enzymes that are strongly induced during apple infection: a
392 serine-protease PEX2_106910, an asparaginyl-peptidase PEX2_039390, an aspartic-protease
393 PEX2_009280, and a subtilin-like serine-protease PEX2_027670. Although, a homologue of
394 PEX2_009280 was reported to have no effect on the pathogenicity of *Leptosphaeria maculans* on

canola (Idnurm et al. 2003), this does not rule out that it may have an important functional role in the virulence in *P. expansum*, especially since the two pathogens exhibit very different modes of infection. Catabolic enzymes that were identified as homologues of proteins affecting pathogenicity are an amidase (PEX2_107240), an aspartic protease (PEX2_085170), and a phospholipase (PEX2_099670) with homology to *Candida albicans* effectors, a metallo-protease (PEX2_089670) homologous to an effector in the barley powdery mildew fungus *Blumeria graminis f. sp. hordei* (Pliego et al. 2013), and a lipase (PEX2_019000) with homology to an autophagy-like lipase, FgATG15, in *F. graminearum*, whose disruption was reported to severely attenuate the infection of wheat heads (Nguyen et al. 2011). Three catabolic enzymes with a predicted NLS were also detected: subtilase (peptidase S8) (PEX2_027670), abhydrolase (PEX2_061790), and an alginate lyase (PEX2_021730).

As previously indicated, gene-products possessing at least two of the five pathogenicity-factor characteristics noted above were selected as top-candidates (Table 1). Among 17 top-candidates, one was a proteolytic enzyme, peptidase S8 (PEX2_027670) that had four of the requisite characteristics designated in the pipeline. PEX2_027670 has a NLS, a peptidase inhibitor I9 domain, and is highly expressed *in planta*. PEX2_027670 also has high homology to a subtilisin-like serine-protease (SPM1) in *M. oryzae*, which functions in autophagy and is required for pathogenicity (Saitoh et al. 2009). Targeted disruption of *SPM1* was shown to affect a variety of infection processes, including spore germination, appressorium formation, host invasion, and post-invasive growth. Moreover, the putative effector AG1IA_07795 of *Rhizoctonia solani* possesses peptidase inhibitor I9 and peptidase S8 domains. Injection of AG1IA_07795 protein into leaves of rice, maize, and soybean leaves was shown to induce cell death (Zheng et al. 2013). Based on the characteristics of PEX2_027670 (denoted as PePRT) and its homology to proteins that are

associated with virulence in other host-pathogen systems, further studies have been done in order to characterize the potential role of this gene in the virulence of *P. expansum* towards apples.

3.3 Characterization of *PePRT*

Peprt encodes a predicted 496 aa protein. Sixteen aa at the N-terminus of the protein represent a signal peptide as predicted by SignalP software. TargetP analysis indicated that PePRT is a secreted protein. PePRT exhibits a high amino acid sequence similarity to a variety of fungal vacuolar serine proteases (Table S7, Fig. S1A), as well as to Proteinase B in *Saccharomyces cerevisiae*.

InterPro analysis of PePRT indicates that it belongs to the Peptidase S8, subtilisin-related family (IPR015500) of proteins. The S8 family of subtilisins is a heterogeneous group of proteins with broad taxonomic distribution. Among S8 family members are numerous secreted alkaline and serine proteases from various fungi, as well as a vacuolar protease B (cerevisin) from *S. cerevisiae*. The members of this family share a characteristic catalytic triad peptide (Asp, His and Ser) (Siezen and Leunissen 1997). PePRT possesses an Inhibitor_I9 domain (PF05922), as well as a Peptidase_S8 domain (PF00082) (Fig. S1B). The majority of subtilases are synthesized as pre-proenzymes that are subsequently translocated through a cell membrane via the pre-peptide (or signal peptide), and finally activated by cleavage of the pro-peptide. The Inhibitor_I9 domain at the N terminus of peptidases is a propeptide domain in MEROPS family S8A members, which is responsible for the modulation of folding and the activity of the pro-enzyme. (Li et al. 1995) [PMID: 7559646].

The expression level of *prt* in PEX2 during apple infection and *in vitro* growth was examined using RT-qPCR (Fig. 2). Results indicated that *Peprt* was expressed in spores and during infection and

Figure 2: Relative expression of *Peprt* in vitro and during apple infection. Bars indicate standard error.

decay development on fruit, as well as during fungal growth *in vitro*. In general, effectors of various plant pathogens have been reported to be expressed solely during host colonization (Arenas et al. 2010, Dallal Bashi et al. 2010, Motteram et al. 2009, Santhanam et al. 2013), however, putative LysM effectors in *P. expansum* were shown to be expressed both during the colonization of apples and during *in vitro* growth (Levin et al. 2017). Notably, relatively high levels of *Peprt* expression (4.5 fold increase) were measured at 24 h after inoculation of fruit wounds (Fig. 2) indicating its possible involvement in the early infection processes in which the *P. expansum*

mycelia starts to invade and colonize fruit tissue. High levels of expression of effectors during early stages of infection prior to the appearance of symptoms have been reported for several plant pathogens. For example, expression levels of NEP1-like, SnTox and LysM effectors in various fungi were highest slightly before or with the appearance of disease symptoms (Arenas et al. 2010, Bolton et al. 2008, Dallal Bashi et al. 2010, Liu et al. 2012, Marshall et al. 2011, Motteram et al. 2009, Santhanam et al. 2013, van der Does et al. 2008).

3.4 Functional analysis of PePRT

Knockout mutants of *P. expansum* PEX2 were constructed using *Agrobacterium tumefaciens*–mediated transformation (Buron-Moles et al. 2012) in order to further investigate the role of PePRT in the pathogenicity and virulence of the fungus. Figure S2A presents the illustration of the wild-type and the respective gene-disrupted loci. Correct T-DNA integration was verified by three PCR (Fig. S2B): 1) using primer pair specific for hygromycin resistance gene which is present in both null mutants and ectopic transformants, but absent in the wild-type (WT) 2) using primer pair to amplify the target gene which is present in the WT and ectopic mutants, but absent in the null mutants, and 3) to examine the accurate insertion of the T-DNA amid the promoter and the terminator of the target gene. The reactions were done using one primer from the hygromycin resistance cassette and another primer located in the genome approximately 150 bases upstream or downstream of the promoter or terminator, respectively. Results indicated that specific PCR products were obtained only in null mutants with correct insertions (Fig. S2B). A knockout mutant ($\Delta Peprt$), containing a single T-DNA integration, and an ectopic transformant possessing the wild-type (WT) gene were selected for further analysis.

470 Radial growth rate of the $\Delta Peprt$ null mutant was slightly lower compared to the WT strain and
 471 the ectopic mutant (Fig. 3A). Disrupted genes coding for proteins homologous to PePRT in *V.*
 472 *dahliae* and *Aspergillus fumigatus* also exhibited a reduction in the radial growth rate (He et al.
 473 2015, Reichard et al. 2000).

Figure 3: Radial growth (A) and colony morphology at 7 dpi (B) on PDA medium of PEX2 (WT), ectopic (E) and deletion ($\Delta Peprt$) mutants of $\Delta Peprt$ in *Penicillium expansum*. Radial growth results are an average of three independent measurements. Bars indicate standard error.

475 Growth studies also indicated that the colony morphology of the $\Delta Peprt$ null mutant was different
476 from that of the WT and the ectopic mutant (Fig. 3B). The mycelia of the $\Delta Peprt$ mutant appeared
477 less dense and the secretion of a brown metabolite/s was observed in the growth medium of mutant
478 cultures growing on PDA (Fig. 3B). In addition to a lower level of mycelial density, color change,
479 and slow growth rate *in vitro*, there was significant reduction in production of conidia (Fig. 4A).
480 Formation of conidia in the mutant was found to be less than 0.2 % of the number of conidia
481 produced by the WT strain (Fig. 4B).

Figure 4: Effect of *Peprt* target deletion on spore production. Color change of the mature colonies (A), spore production (B) of PEX2 (WT), ectopic (E) and deletion ($\Delta Peprt$) mutants of *Peprt*. Sporulation was calculated after 11 d growth. Results are an average of three independent measurements. Bars indicate standard error.

483 SEM images of ten-day-old mutant colonies confirmed a severe disruption in spore production
484 (Fig. 5).

Figure 5: Scanning electron microscopy image of 10 d old colony grown on PDA plates. Conidiophores of the $\Delta Peprt$ mutant are marked by the white circles in the X300 magnification.

486 While the mycelia in the WT strain was heavily covered with conidiophores carrying long chains
 487 of spores, the, mutant cultures had a much lower number of conidiophores with short chains of
 488 spores (Fig. 6). Relative to the WT, hyphae in the mutant was observed to be significantly wider
 489 in diameter, with no visible vacuoles (Fig. 6A).
 490

Figure 6: Confocal microscopy image (A) and staining of the vacuole membrane by FM4-64 (B) of one-week old hyphae. PEX2 (WT) and deletion ($\Delta Peprt$) mutants of *Peprt*.

FM dye that specifically labels membrane-bound organelles was used to further visualize cell contents. In contrast to the WT strain, which had large distinctive vacuoles, the hyphae of the $\Delta Peprt$ mutant were strongly stained with FM implying the presence of numerous small vesicles (Fig. 6B). The observed differences could be due to impaired autophagy or endocytosis. In research conducted with a *Spm1* coding vacuolar-localized protease, a gene in *M. oryzae* that is homologous to *Peprt*, spherical vacuoles developed in both the WT and the *spm1* mutant, however, while WT vacuoles were transparent and contained few detectable particles, granular particles were always observed in the putative vacuoles of the *spm1* mutant. This indicates that the vacuolar components were not degraded in the mutant, resulting in the accumulation of granular structures. Surprisingly, internalization of FM4-64 was not observed in conidia of the *spm1* mutant (Saitoh et al. 2009). To further examine the effect of *Peprt* deletion on the germination process, visual examination of germinating spores in the WT, mutant, and ectopic strains was conducted using light microscopy. No significant difference in the percent germination of spores in the WT and mutant strains was observed after 18 h of incubation on 1.5 % agar plates (Fig. 7A). Therefore, the *Peprt* deletion did not appear to have any apparent effect on the germination process. Germinating spores in the mutant, however, were much larger and the germ-tubes arising from the spores were thicker and longer than in the WT and ectopic mutant (Fig. 7B). The average germ-tube length in the mutant was more than two-fold greater than in the WT or ectopic mutant (Fig. 7C). These findings, together with the fact that the hyphae in the $\Delta Peprt$ mutant are thicker than in the WT, suggests that the *Peprt* deletion had an effect on cell-growth.

Figure 7: Effect of *Peprt* deletion on spore germination. Percent of germinating spores (**A**), microscope observation of the germination of spores (**B**) and the length of the germ tubes (**C**) 18 h after inoculation on 1.5 % agar plates of PEX2 (WT), ectopic (E) and deletion (Δ *Peprt*) mutants of *Peprt*. Percent of germination is an average of 20 different microscopic fields including 20-30 spores each. Length of the germs is an average of more than 50 measurements. Bars indicate standard error. Different letters indicate significant differences at $P < 0.05$ based on nested one-way ANOVA followed by Tukey's honest significant difference (HSD) test.

513 In general, disruption of the *Peprt* gene had pleiotropic effects manifested by altered mycelial
514 growth and morphology, secretion of brown colored metabolites, and reduced sporulation.
515 Consistent with these results, deletion of Proteinase B homologous genes in *V. dahliae* (Vd991)
516 also resulted in a significant decrease in the production of conidia, significant reduction in growth
517 rate, and a reduction in virulence, relative to the wild-type (He et al. 2015). Targeted disruption of
518 the cellular serine proteinase ALP2-encoding gene in *Aspergillus fumigatus* also resulted in a
519 slightly decreased rate of vegetative growth and a more than 80 % reduction in sporulation in the
520 alp2-negative mutants, which was correlated with an approximate 50 % reduction in the median
521 diameter of conidiophore vesicles (Reichard et al. 2000).

522 Alterations of the vacuolar structure in the $\Delta Peprt$ mutant, together with the high level of
523 homology of PePRT to autophagy-related proteins, such as a vacuolar protease B from *S.*
524 *cerevisiae* and SPM1 from *M. oryzae*, indicates that PePRT may have a functional role in
525 autophagy in *P. expansum*. The increased pigmentation of the mutant cultures growing on PDA
526 plates and the marked reduction in sporulation are consistent with the findings that autophagy in
527 filamentous fungi is involved in cellular processes such as cell differentiation, pathogenicity, and
528 secondary metabolite production (Liu et al. 2016, Voigt and Pöggeler 2013). For example, *atg1*
529 deletion mutants of *P. chrysogenum* that are impaired in autophagy and sporulation, have
530 significantly increased levels of penicillin production (Bartoszewska et al. 2011). The increase in
531 the diameter of hyphae and spore size in the $\Delta Peprt$ mutant, relative to the WT, may provide
532 additional evidence of impaired autophagy since induced autophagy in *Drosophila* cells was
533 shown to reduce cell size (Scott et al. 2007). Furthermore, a starvation-induced reduction in cell
534 size was significantly inhibited in cells unable to undergo autophagy (Hosokawa et al. 2006).

Associated with this response, mycelia in a Δ Aoatg8-1-1 mutant of *A. oryzae*, that was defective in autophagy, was observed to be thicker, relative to the WT (Kikuma et al. 2006).

The fact that PePRT possesses a signal peptide and is predicted to be secreted is not in contradiction to its potential role in the vacuole. An examination of the derived amino acid sequences of secreted and vacuolar plant proteins indicated that they all have an amino terminal domain conforming to a eukaryotic signal sequence, indicating that there are no vacuole-specific and secretion-specific signal peptides (Chrispeels 1991). In this regard, Reichard et al. (2000) stated that there is a possibility that cellular serine proteinase ALP2 in *A. fumigatus* is a natural component of the vacuole as well as the cell wall. Moreover, overexpression of the vacuolar proteinase PrA in *S. cerevisiae*, and the overexpression of the corresponding *A. fumigatus* enzyme, PEP2, in the yeast, *Pichia pastoris*, resulted in the enzymes being secreted and thus present in the yeast culture supernatants (Rothman and Stevens 1986).

‘Golden Delicious’ apples were inoculated with spores suspension obtained from cultures of the WT, ectopic and null mutants in order to examine the effect of *Peprt* deletion on pathogenicity. After inoculation, the percent infection and the rate of lesion development were recorded, starting at 3 d post-inoculation (dpi). No significant differences in disease incidence were found (data not shown), however, lesion diameter in apples inoculated with the Δ *Peprt* mutant was approximately 20 % smaller throughout the measurement time span than in the WT and the ectopic mutant (Fig. 8).

Figure 8: The effect of the *Peprt* deletion on virulence of PEX2 on apples as expressed by rate of decay development on Golden Delicious apples and compared to that of the WT and the ectopic (E) mutant. Progression of rot development was expressed as lesion diameter (cm) at different times after inoculation (dpi).

Results are an average of three independent measurements. Bars indicate standard error. Asterisks indicate significant differences at $P < 0.05$ based on nested one-way ANOVA followed by Tukey's honest significant difference (HSD) test.

Attenuation of the virulence of $\Delta Peprt$ could be a consequence of impaired autophagy as has been reported in another pathogen-plant pathosystem (Liu et al. 2016, Voigt and Pöggeler 2013). Inoculation of seedlings of rice and barley with a *spmI* mutant strain of *M. oryzae* resulted in reduced disease symptoms, relative to inoculation with the WT strain, at 7 d after inoculation (Saitoh et al. 2009). At present, the possibility that PePRT is secreted and has direct functional role in host-pathogen interaction, however, cannot be ruled out. In fact, *AGIIA_07795* a gene encoding a peptidase S8 with peptidase inhibitor I9 domain from *R. solani* was shown to cause cell death when the protein was injected into rice, maize and soybean leaves (Zheng et al. 2013).

4. Conclusions

The results presented in this study provide for the first time a description of the complete secretome of *P. expansum* that has been used to construct a pipeline for prediction of putative genes with potential role in virulence. The approach was based on bioinformatics tools and experimental data. We used known effector features, transcriptomic information and homology to fungal proteins reported in the literature to have role in virulence to identify putative effectors in *P. expansum* genome. A total of 533 genes encoding secreted proteins were identified, representing 5.0 % of the *P. expansum* PEX2 proteome. Using the transcriptomic data obtained during infection and decay development of *P. expansum* on apple fruit, the number of secretome-related genes was reduced to 297 that are actively transcribed. Among these, 103 genes (35 % of the total predicted *in planta* secretome) code for hydrolytic enzymes. The majority of the secreted enzymes are carbohydrate-degrading enzymes among which five coding for pectin-degrading enzymes are highly induced during the infection and decay of apple fruit by *P. expansum*, indicating that they may represent an important aspect of pathogenicity and virulence. Applying the pipeline we have

predicted 17 genes that are likely involved in pathogenicity/virulence. One of the top-candidates is a proteolytic enzyme highly expressed in planta, a subtilisin-related peptidase, S8 (PePRT) which had four of the requisite characteristics designated in the pipeline.

$\Delta Peprt$ mutants were shown to have attenuated virulence on apples as well as lower growth rate, less dense mycelia, lower number of conidiophores with shorter conidia-chains compared to the WT. Moreover, the germinating spores of the mutant were significantly larger than that of the WT. The hyphae of $\Delta Peprt$ were wider compared to the WT and had disrupted vacuolar structure. Based on these findings and the homology of the PePRT to autophagy-related proteases, PePRT potentially may play dual role in autophagy or as a virulence factor. Either way it does appear to play a functional role in virulence of *P. expansum* on apples.

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Supporting Information legends

Figure S1: Deletion of *PePrt* in *Penicillium expansum* PEX2. (A) Diagram of the wild type (WT) and deleted *Peprt* loci. The hygromycin selectable marker present in the T-DNA of plasmids pRFHU2-PePrt replaces the corresponding *Peprt* gene by homologous recombination, to generate the Δ *Peprt* null mutant (B) Polymerase chain reaction amplification of the wild type, Δ *Peprt* null mutants and their respective ectopic (E) mutants with diagnostic primers. In section (B), the first two images (from left) were cropped to remove lanes that were not related to the content of the text.

Table S1: Primers used in the study

Table S2: Small, Cysteine-rich proteins with unknown function.

Table S3: Proteins with known effector-domains

Table S4: NLS-carrying proteins

Table S5: Genes homologous to effectors found in PHI database.

Table S6: Degrading enzymes.

A

As_fum	-----MKGYLSLSILPLLVAAFPVVDSIHNGAAPILSSMNAKEVP	41
Cl_her	-----MRGALAGLSLATLATASFVLVNSIHNDAAPIISASNAKEIA	41
Pe_chr	-----MKGFLSLTLLPLLVAAFPVAVNSIHNDAAPIISMTSKDIP	41
Pe_exp	-----MKGFLSLTLLPLLVAAFPVAVNSIHNDAAPIISMTSKDIP	41
Pe_oxa	-----MKGFLSLTLLPLLVAAFPVAVNSIHNDAAPIISMTSKDIP	41
Sa_cer	PHHKGCGCHENKVEEKKVKGKVKGKKHHEKTLKGRHHNRLLPLVSTAQFNPDAISKIIP	180
Ve_dah	-----MRVIAAVAVAAVVSATPSFSSETIHRDAAPILSSSNAEAI	42
Ma_ory	-----MKSVILLSLAACVAAPTAGVETIHDGAAPILSSSNAEAI	41
As_fum	DSYIVVFKKHVNA-ESAAAHHSW---VQDIHSAQNERV-ELRKRSLFGFGEAYLGLKN	95
Cl_her	INYMIFKFKHVTQ-NLAAEHHSW---VQDLHEKTQVAKTELKRSSQSMVDDIFNGLKH	96
Pe_chr	DSYIVVFKKHVDP-SSASAHQSW---LQEVHTAHTGRM-ELKGRSLFGFDFEAFNGLKH	95
Pe_exp	DSYIVVFKKHVDE-SSASAHQSW---LQEVHTAHTGRM-ELKGRSLFGFDFEAFNGLKH	95
Pe_oxa	DSYIVVFKKHVGA-SAASAHHSW---VQDIHSDNV-RM-ELKGRSLFGFESEFYLVGVKH	94
Sa_cer	NRYIIVFKRGAPEEIDFKKENVQQAQLQSVENLSAEDAFFISTKDTSLSTSEAGGIQDS	240
Ve_dah	DSYIVFKDKHVSSEASDHHSWV---QKIHDSKGAERL-ELRKRQGEPIVETTFEGLKH	97
Ma_ory	NAYIIFKFKHVDHKSADHQMWI---QKVHGEREDERL-ELRKRGLFDSVNDFTGLKH	96
As_fum	TFDIAGSLVGYSGHFHEDVIEQVRKHPDVEYIEKDSEVHTMEDPTV-----EK	143
Cl_her	TYNIAGGLMGYAGHFDEVDIEQIRRHDPDVEIREDQEVHVLGSESEV-----EK	145
Pe_chr	TFHIAGSLGAGYAGHFHEDVIEQIRRHDPDVEYIEKDSEVRTMSEGSV-----EK	143
Pe_exp	TFHIAGSLGAGYAGHFHEDVIEQIRRHDPDVEYIEKDSEVRTMSEGSV-----EK	143
Pe_oxa	TFHIAGSLGAGYAGHFHEDVIEQIRRHDPDVEYIEKDSEVHHFEDPAI-----EK	142
Sa_cer	FNIDNLFSGYIGYFTQETVDLIRQNPLVDFVERDSIVEATEFDTQNS-----287	
Ve_dah	TYTLG-GFLGYSGHFDEATIEEVRKHADVEFVKDSIVHTM/FVQTEAQTEDKCDEGEVK	156
Ma_ory	TYNVSGLGYAGHFDEETIEKVRRHDPDVEAIEDTIVHTMRYEEVKKDECPDNL---EK	153
As_fum	SAPFWGLARI SHRDSLSFGTFNKYLYASEGGEGVDAYTIDTGINVDHVD FEGRAGWGTIP	203
Cl_her	NAPFWGLARI SHRDSLSFGTFNKYLYEDDQEGVDYVYVDTGTNVVDHVD FEGRASWGTIP	205
Pe_chr	NAPFWGLARI SHRESLSFGTFNKYLYAEEGGEGVDAYVIDTGANVVKHVD FEGRANWGTIP	203
Pe_exp	NAPFWGLARI SHRESLSFGTFNKYLYAEEGGEGVDAYVIDTGANVVKHVD FEGRATWGTIP	203
Pe_oxa	NAPFWGLARI SHRDSLSFGTFNKYLYAEDGGEGVDAYVIDGTNVVDHVD FEGRASWGTIP	202
Sa_cer	-APFWGLARI SHRERLNLGSFNKYLYDDDAGRGTYSYVIDGTNVINHKDFEKRATWGTIP	346
Ve_dah	SAPFWGLRVSHRESLNFNKNYLYFADNAGEGVDAYIIDTGTNVVDHVD FEGRAGWGTIP	216
Ma_ory	GAPFWGLRVSHRESLFSFTYKNYLYSAEGGEGVDAYVIDGTNIDHVD FEGRAGWGTIP	213
As_fum	TDDEADGNGHGTCHCSGTIAGRKYGVAKKANLYAVKVLRSNGSGTMSDVVAGVEWVKSH	263
Cl_her	QGDADEDGNGHGTCHCSGTIVAGKKYGVAKKAHVAVKVLRSNGSGTMSDVVKGVEYAAESH	265
Pe_chr	QGDADEDGNGHGTCHCSGTIAGKKFGVAKKANVYAVKVLRSNGSGTMSDVVKGVEWAAEAH	263
Pe_exp	QGDADEDGNGHGTCHCSGTIAGKKFGVAKKANVYAVKVLRSNGSGTMSDVVKGVEWAAEAH	263
Pe_oxa	QGDDVDGNGHGTCHCSGTIAGKKYGVAKKANVYAVKVLRSNGSGTMSDVVKGVEWAAEAH	262
Sa_cer	LNDEDLGNGHGTCHCAGTIASKHYGVAKKANVAVKVLRSNGSGTMSDVVKGVEYAAKAH	406
Ve_dah	QGDEDVDGNGHGTCHCSGTIVAGSKYGVAKKAHVAVKVLRSNGSGTMSDVVKGVEWAAQSH	276
Ma_ory	ANDQIDGNGHGTCHCSGTIVAGKKYGVAKKAQYVAVKVLRSNGSGTMSDVVAGVDFAAKSH	273
As_fum	LKKVKDAKDKGKIRGFKGSVANMSLGGGKSRITLDAVNAGVEAGLHFVAAGNDNADACNY	323
Cl_her	LEQVSIITKKGKRGFKGSTANMSLGGGKSPILDKAVNAAVDAGIHFVAAGNDNADACNY	325
Pe_chr	IKKSKKGDKK---FKGSVANMSLGGGSRITLDAVNAAVDAGIHFVAAGNDNADACNY	319
Pe_exp	IKKSKKGDKK---FKGSVANMSLGGGSRITLDAVNAAVDAGIHFVAAGNDNADACNY	319
Pe_oxa	IKKSKAAKDGKAGFKGSVANMSLGGGSRITLDAVNAAVDAGMHFAVAAGNDNADACNY	322
Sa_cer	QKEAQEKKGK---FKGSTANMSLGGGKSPALDVAVNAAVEAGIHFVAAGNDNADACNY	462
Ve_dah	LEQVKKAKDGKRGFKGSVANMSLGGGKTTALDRVVDAAVEAGIHFVAAGNDNADACNY	336
Ma_ory	KQVSAKADGKRGFKGSVANMSLGGGKTTLLDAVNAAVDAGIHFVAAGNDNADACNY	333
As_fum	SPAAAEAVITVGASTLQDERAYFSNYGKCTDIFAPGLNISTWIGSKYAVNTISGTSMAS	383
Cl_her	SPAAAEAVITVGASTLQDERAYFSNYGKCTDIFAPGLNIQSTWIGSKYAVNTISGTSMAS	385
Pe_chr	SPAAAEKAVITVGASTLQDERAYFSNYGKCTDIFAPGLNISTWIGSDHATNTISGTSMAS	379
Pe_exp	SPAAAEKAVITVGASTLQDERAYFSNYGKCTDIFAPGLNISTWIGSEHATNTISGTSMAS	379
Pe_oxa	SPAAAEKAVITVGASTLQDERAYFSNYGKCTDIFAPGLNISTWIGSKYAVNTISGTSMAS	382
Sa_cer	SPASADKAVITVGASTLQDERAYFSNYGKCTDIFAPGLNISTYIGSDDATATISGTSMAS	522
Ve_dah	SPAAAQAVITVGASTLQDERAYFSNYGKCTDIFAPGLNISTWIGSKYATNTISGTSMAS	396
Ma_ory	SPAAAQAVITVGASTLQDERAYFSNYGKCTDIFAPGLNIQSTWIGSKYATNTISGTSMAS	393
As_fum	PHIAGLLAYFVSLQPSKDSA---FAVDELTPKGLKGDIIATATQGALTDIPSDTNNLLAW	440
Cl_her	PHVAGLLAYFVSLQPSKDSA---FAVADISPKGLKANLISATVGAALTDVPSNTANILAW	442
Pe_chr	PHIAGLLAYFVSLQPSKDSA---YAVADVTPKQLKAALISVATEGTLTDIPSDTNNLLAW	436
Pe_exp	PHIAGLLAYFVSLQPSKDSA---YAVADVTPKQLKAALINVATQGALTDIPSDTNNLLAW	436
Pe_oxa	PHIAGLLAYFVSLQPSKDSA---YAVEEITPKGLKDALITATSGALSDIPSDTNNLLAW	439
Sa_cer	PHVAGLLAYFVSLQPSKDSA---YAVADVTPKQLKAALINVATQGALTDIPSDTNNLLAW	582
Ve_dah	PHICGLLAYFVSLQPSKDSA---YVAPITPKGLKANLIVATGIGALSGIPSDTNNLLAW	453
Ma_ory	PHIAGLLAYFVSLQPSKDSA---YSLATITPEKGLKADLIKVGTGILTDIPKDTNNLLAW	450

B

Figure S1: Multiple alignment of PePRT with fungal vacuolar serine proteases and proteinase B from *S. cerevisiae* (A) and diagrammatic representation of its structure (B). Purple rectangle—signal peptide, orange lines mark amino acids from the active site. Amino acids marked by orange flags form the catalytic triad.

Figure S1. Deletion of *PePrt* in *Penicillium expansum* PEX2. (A) Diagram of the wild type (WT) and deleted *Peprt* loci. The hygromycin selectable marker present in the T-DNA of plasmids pRFHU2-*PePrt* replaces the corresponding *Peprt* gene by homologous recombination, to generate the $\Delta Peprt$ null mutant (B) Polymerase chain reaction amplification of the wild type, $\Delta Peprt$ null mutants and their respective ectopic (E) mutants with diagnostic primers. In section (B), the first two images (from left) were cropped to remove lanes that were not related to the content of the text.

Table S1: Primers used in the study

Name	Sequence	Explanation
Gene expression analysis		
PepI_F	GGT GAT GCC GAC GAG GAT GG	
PepI_R	CCC GAG CCG TTA GAG CGA AG	
28S_F	GGA ACG GGA CGT CAT AGA GG	
28S_R	AGA GCT GCA TTC CCA AAC AAC	
Knockout mutant construction and analysis		
Prt_Prom_F	ggtcttaaUCG GCA CTT CCA CTG TCTC	
Prt_Prom_R	ggcattaaUAT GAT GCA AGC GGG TAT TAC	
Prt_Ter_F	ggacttaaUAT ACA TGG ATA AGT TCG GAC TGA	
Prt_Ter_R	gggttttaaUGC ATC GGA ATT TGT TTG ATAG	
RF-1	AAA TTT TGT GCT CAC CGC CTG GAC	Confirmation of terminator fusion into pRFHU2 plasmid
RF-6	ACG CCA GGG TTT TCC CAG TC	Confirmation of terminator fusion into pRFHU2 plasmid
RF-2	TCT CCT TGC ATG CAC CAT TCC TTG	Confirmation of promoter fusion into pRFHU2 plasmid
RF-5	GTT TGC AGG GCC ATA GAC	Confirmation of promoter fusion into pRFHU2 plasmid
HMBF1	CTG TCG AGA AGT TTC TGA TCG	
HMBR1	CTG ATA GAG TTG GTC AAG ACC	

Prt_center_F	CAA ATT GCT GGA TCA CTC AC	1*
Prt_center_R	GTT AGA GCG AAG AAC CTT GAC	2*
Prt_check_F	GGT AAA TTG CTT TCT CCC TGT CTG AC	3*
Prt_check_R	CGG TTC TGA TAC ATT CGA TAG ACA	4*
HPH1F	ACG AGG TCG CCA ACA TCT TCTTCT	5*
HPHPRO4	GCA CCA AGC AGC AGA TGA TA	6*

Determination of T-DNA copy number

PeTub_1F	AGC GGT GAC AAG TAC GTT CC
PeTub_2R	ACC CTT AGC CCA GTT GTT AC
Prt_RT_F	ATG ATG TGA TGG GCT TCT CC
Prt_RT_R	ATG ATG TGA TGG GCT TCT CC

*Numbers 1-6 refer to primers in Fig. S1

Table S2: Small, Cysteine-rich proteins with unknown function.

Gene name	size (aa)	# Cys	High exp. (hpi)
PEX2_000510	170	8	
PEX2_002470	273	5	24, 48, 72
PEX2_006020	182	4	
PEX2_006280	149	5	
PEX2_007820	103	6	
PEX2_011820	221	16	
PEX2_020550	173	8	
PEX2_025800	194	6	
PEX2_026210	213	4	
PEX2_035430	276	17	
PEX2_038290	138	10	48, 72
PEX2_056120	271	5	
PEX2_056460	176	4	spores
PEX2_063200	148	5	
PEX2_064300	259	4	
PEX2_074940	224	4	
PEX2_079250	122	7	
PEX2_080310	95	6	
PEX2_080970	244	7	
PEX2_085640	263	6	
PEX2_088490	286	9	
PEX2_090640	270	11	
PEX2_092040	297	4	24
PEX2_095180	159	8	
PEX2_108080	79	8	

Table S3: Proteins with known effector-domains

Gene name	annotation	NLS	PHI	High expression (hpi)
			http://www.phi-base.org/	
PEX2_002570	PF01822.14	WSC		
PEX2_012920	PF03067	chitin-binding		
PEX2_021580	PF03388.8	L-like lectin		
PEX2_027670	PF05922.11; PF00082.17	Inhibitor_I9; Peptidase_S8	V 2117, <i>M.oryzae</i>	48, 72
PEX2_028650	PF01828.12	Peptidase A4		
PEX2_041810	PF03388.8	L-like lectin		
PEX2_043920	PF05730	CFEM		
PEX2_046410	PF02018.12	carbohydrate-binding		
PEX2_053590	IPR018392	LysM		
PEX2_064530	IPR018392	LysM		
PEX2_065330	PF07249.7	Cerato-platanin	3164, <i>T. virens</i>	
PEX2_080220	PF05630.6	NPP-1	2342, <i>B. elliptica</i>	24, 48, 72
PEX2_091440	PF01476	LysM		
PEX2_092030	PF05730	CFEM		
PEX2_105000	PF01822.14	WSC		72

(PHI data: white - not available, grey – no effect on pathogenicity, yellow - mixed output, green – reduced pathogenicity, purple – lethal)

Table S4: NLS-carrying proteins

Gene name	annotation	PHI http://www.phi-base.org/	High expression (hpi)
PEX2_010420	PF00149.23 Metallophos		
PEX2_017860	PF00069.20; Pkinase; PF06479.7 Ribonuc_2-5A	1225; <i>Gibberella_zeae</i>	
PEX2_018330			
PEX2_021730	PF05426.7 Alginate_lyase	1846; <i>Gibberella_zeae</i>	
PEX2_027670	PF05922.11; Inhibitor_I9; PF00082.17 Peptidase_S8	2117; <i>Magnaporthe_oryzae</i>	48, 72
PEX2_035730	IPR013320 Concanavalin A-like lectin/glucanase domain		
PEX2_047400	PF00085.15 Thioredoxin	2644; <i>Salmonella_enterica_serovar_Typhimurium</i>	
PEX2_051040	PF00226.26 Heat shock protein DnaJ		
PEX2_052860	PF04082.13 Fungal_trans	1800; <i>Gibberella_zeae</i>	
PEX2_056540	PF13639.1 Zinc finger, RING-type	2401; <i>Xanthomonas_campestris_pv_vesicatoria</i>	24
PEX2_061790	PF12697.2 Abhydrolase_6		
PEX2_071030			
PEX2_076710	PF00012.15 HSP70	2058; <i>Magnaporthe_oryzae</i>	
PEX2_084770	PF03896.11 TRAP_alpha (translokon associated protein)		24
PEX2_085110			
PEX2_087840	PF00328.17 Histidine phosphatase superfamily, clade 2		
PEX2_087920	PF00491.16 Arginase		
PEX2_096760	IPR015919 Cadherin-like		
PEX2_106730	PF01019.16; G_glu_transpept; PF00730.20 HhH-GPD	2411; <i>Sclerotinia_sclerotiorum</i>	

(PHI data: white - not available, grey – no effect on pathogenicity, yellow - mixed output, green – reduced pathogenicity, purple – lethal)

Table S5: Genes homologous to effectors found in PHI database.

Gene name	Annotation	PHI	NLS	High exp. (hpi)
PEX2_000290	PF07732.10; PF00394.17; PF07731.9	2921 <i>Colletotrichum graminicola</i>		
PEX2_000950	PF13802.1; PF01055.21	1071 <i>Ustilago maydis</i>		
PEX2_006870	PF07732.10; PF00394.17; PF07731.9	2700 <i>Colletotrichum orbiculare</i>		
PEX2_010260	PF00332.13	61 <i>Candida albicans</i>		
PEX2_013190	PF01095.14	1028 <i>Botrytis cinerea</i>		
PEX2_016450	PF00544.14	222 <i>Colletotrichum gloeosporioides</i>		
PEX2_017860	PF00069.20; PF06479.7	1225 <i>Gibberella zeae</i> (related: <i>Fusarium graminearum</i>)	V	48, 72
PEX2_019000	PF01764.20	1166 <i>Gibberella zeae</i> (related: <i>Fusarium graminearum</i>)		
PEX2_022780	PF01565.18	1046 <i>Cercospora nicotianae</i>		
PEX2_027670	PF05922.11; PF00082.17	2117 <i>Magnaporthe oryzae</i> (related: <i>Magnaporthe grisea</i>)	V	
PEX2_030850	PF00933.16; PF01915.17; PF14310.1	24 <i>Gaeumannomyces graminis</i>		
PEX2_031340	PF00295.12	1027 <i>Botrytis cinerea</i>		

PEX2_031470	PF00544.14	Pec_lyase_C	3226 <i>Penicillium_digitatum_</i>		
PEX2_032490	PF13561.1;P F00109.21;P F02801.17	adh_short_C2;ketoacyl- synt;Ketoacyl-synt_C	96 <i>Candida_albicans</i>		
PEX2_036960	PF01565.18; PB001685;P F08031.7	FAD_binding_4;BBE	1046 <i>Cercospora_nicotianae</i>		
PEX2_037380	PF00254.23	FKBP_C	2305 <i>Botrytis_cinerea</i>		
PEX2_039220	PF07732.10; PF00394.17; PF07731.9	Cu-oxidase	2700 <i>Colletotrichum_orbicul are</i>		
PEX2_042850	PF13802.1;P F01055.21	Gal_mutarotas_2;Glyco _hydro_31	1071 <i>Ustilago_maydis</i>		
PEX2_044440	IPR001579	Glycoside hydrolase chitinase active site	2388 <i>Metarhizium_anisopliae</i>		
PEX2_047400	PF00085.15; PB015947	Thioredoxin	2644 <i>Salmonella_enterica_se rovar_Typhimurium</i>	V	
PEX2_048400	PF03198.9;P F07983.8	Glyco_hydro_72;X8	2808 <i>Fusarium_oxysporum_f _sp._Lycopersici</i>		48, 72
PEX2_051820	PF01565.18; PB012605;P B003699	FAD_binding_4	1046 <i>Cercospora_nicotianae</i>		
PEX2_052110	PF03636.10; PF03632.10; PF03633.10	Glyco_hydro_65N;Glyc o_hydro_65m;Glyco_h ydro_65C	3076 <i>Candida_parapsilosis_</i>		
PEX2_054250	PF00331.15; PF00734.13	Glyco_hydro_10;CBM _1	2208 <i>Magnaporthe_oryzae_(r elated:_Magnaporthe_g risea)</i>		
PEX2_056540	PB005982;P F13639.1	zf-RING_2	2401 <i>Xanthomonas_campestr is_pv_vesicatoria</i>	V	24

PEX2_065330	PF07249.7	Cerato-platanin	3164 <i>Trichoderma_virens_</i>
PEX2_067560	PF00457.12	Glyco_hydro_11	546 <i>Botrytis_cinerea</i>
PEX2_070420	PF00704.23	Glyco_hydro_18	2388 <i>Metarhizium_anisopliae</i>
PEX2_076170	PF00454.22; PF02260.15	PI3_PI4_kinase;FATC	1224 <i>Gibberella_zeae_(related:_Fusarium_graminearum)</i>
PEX2_076710	PF00012.15	HSP70	2058 <i>Magnaporthe_oryzae_(related:_Magnaporthe_grisea)</i>
PEX2_081580	PF00328.17	His_Phos_2	3236 <i>Cryptococcus_neoformans_</i>
PEX2_082100	PF00067.17	p450	438 <i>Botrytis_cinerea</i>
PEX2_085170	PF00026.18	Asp	126 <i>Candida_albicans</i>
PEX2_086800	PF00085.15; PF00085.15; PF07749.7	Thioredoxin;Thioredoxin;ERp29	2644 <i>Salmonella_enterica_serovar_Typhimurium</i>
PEX2_088440	PF01565.18	FAD_binding_4	1046 <i>Cercospora_nicotianae</i>
PEX2_089670	PF13933.1	HRXXH	2897 <i>Blumeria_graminis_f._sp._hordei</i>
PEX2_093030	PF07335.6	Glyco_hydro_75	2403 <i>Fusarium_solani</i>
PEX2_097030	PF07732.10; PF00394.17; PF07731.9	Cu-oxidase_3;Cu-oxidase;Cu-oxidase_2	2920 <i>Colletotrichum_graminicola</i>
PEX2_097510	PF01055.21	Glyco_hydro_31	1071 <i>Ustilago_maydis</i>
PEX2_098230	PF01793.11	Glyco_transf_15	3096 <i>Beauveria_bassiana_</i>
PEX2_099670	PF01735.13	PLA2_B	105 <i>Candida_albicans</i>

V

PEX2_099890	PF00150.13	Cellulase	323 <i>Verticillium_fungicola</i>	V	24, 48
PEX2_104640	IPR017853	Glycoside hydrolase	816 <i>Magnaporthe_oryzae</i>		
PEX2_106730	PF01019.16; PF00730.20	G_glu_transpept;HhH-GPD	2411 <i>Sclerotinia_sclerotiorum</i>		
PEX2_107230	PF00544.14	Pec_lyase_C	3226 <i>Penicillium_digitatum_</i>		
PEX2_107240	PF01425.16	Amidase	2654 <i>Candida_albicans</i>		
PEX2_108490	PF00085.15; PF13848.1	Thioredoxin;Thioredoxin_6	2644 <i>Salmonella_enterica_serovar_Typhimurium</i>		

(PHI data: yellow - mixed output, light green – reduced pathogenicity, dark green - loss of pathogenicity; purple – lethal, brown - effector)

Table S6: Degrading enzymes.

Gene name	Annotation	PHI	High exp. (hpi)
Carbohydrate-degrading			
PEX2_061790	PF12697.2	Abhydrolase_6	<i>Gibberella zeae</i> (related: <i>Fusarium graminearum</i>)
PEX2_068420	PF12697.2	Abhydrolase_6	
PEX2_038250	PF12695.2	Abhydrolase_5	
PEX2_109950	PF12695.2	Abhydrolase_5	
PEX2_021730	PF05426.7	Alginate_lyase	
PEX2_002760	PF00128.19	Alpha-amylase	<i>Verticillium fungicola</i>
PEX2_068560	PF06964.7	Alfa-L-arabinofuranosidase C-terminus	
PEX2_107960	PF06964.7	Alfa-L-arabinofuranosidase C-terminus	
PEX2_092140	PF05592.6	Bacterial alpha-L-rhamnosidase (Glyco_hydro78)	
PEX2_099890	PF00150.13	Cellulase (Glyco-hydro 5)	
PEX2_036500	PF00135.23	COesterase	<i>Magnaporthe oryzae</i> (related: <i>Magnaporthe grisea</i>) <i>Botrytis cinerea</i> <i>Magnaporthe oryzae</i> (related: <i>Magnaporthe grisea</i>) <i>Magnaporthe oryzae</i> (related: <i>Magnaporthe grisea</i>)
PEX2_052910	PF00135.23	COesterase	
PEX2_082800	PF00135.23	COesterase	
PEX2_108390	PF00135.23	COesterase	

PEX2_101220	PF07944.7	DUF1680 (Beta-L-arabinofuranosidase)	
PEX2_000950	PF01055.21; PF13802.1	Glyco_hydro_31; Gal_mutarotas_2	<i>Ustilago_maydis</i>
PEX2_042850	PF01055.21; PF13802.1	Glyco_hydro_31; Gal_mutarotas_2	<i>Ustilago_maydis</i>
PEX2_015850	PF12999.2; PF13015.1	Glucosidase Beta subunit-like	
PEX2_003900	PF00722.16	Glyco_hydro_16	
PEX2_004230	PF00933.16; PF01915.17; PF14310.1	Glyco_hydro_3; Glyco_hydro_3_C; Fn3-like	<i>Ustilago_maydis</i>
PEX2_004580	PF04616.9	Glyco_hydro_43	
PEX2_005800	PF01532.15	Glyco_hydro_47	<i>Aspergillus_fumigatus</i>
PEX2_010260	PF00332.13	Glyco_hydro_17	<i>Candida_albicans</i>
PEX2_011360	PF00933.16; PF01915.17; PF14310.1	Glyco_hydro_3; Glyco_hydro_3_C; Fn3-like	<i>Ustilago_maydis</i>
PEX2_013620	PF00251.15; PF08244.7	Glyco_hydro_32N; Glyco_hydro_32C	
PEX2_016360	PF02838.10; PF00728.17	Glyco_hydro_20b; Glyco_hydro_20	
PEX2_020130	PF01670.11	Glyco_hydro_12	
PEX2_030850	PF00933.16; PF01915.17; PF14310.1	Glyco_hydro_3; Glyco_hydro_3_C; Fn3-like	<i>Gaeumannomyces_graminis</i>
PEX2_031200	PF04616.9	Glyco_hydro_43	
PEX2_031290	PF00722.16	Glyco_hydro_16	
PEX2_031340	PF00295.12	Glyco_hydro_28	<i>Botrytis_cinerea</i>

PEX2_034450	PF03443.9	Glyco_hydro_61	<i>Gibberella_zeae</i> (related: <i>Fusarium_graminearum</i>)	
PEX2_037880	PF00933.16; PF01915.17; PF14310.1	Glyco_hydro_3; Glyco_hydro_3_C; Fn3-like	<i>Septoria_lycopersici</i>	
PEX2_038010	PF14845.1; PF00728.17	Glycohydro_20b2; Glyco_hydro_20		
PEX2_043650	PF00933.16; PF01915.17; PF14310.1	Glyco_hydro_3; Glyco_hydro_3_C; Fn3-like	<i>Ustilago_maydis</i>	
PEX2_044530	PF02055.11	Glyco_hydro_30		
PEX2_047370	PF00722.16	Glyco_hydro_16		
PEX2_048400	PF03198.9; PF07983.8	Glyco_hydro_72; X8	<i>Fusarium_oxysporum</i> f.sp. <i>Lycopersici</i>	48, 72
PEX2_052110	PF03636.10; PF03632.10; PF03633.10	Glyco_hydro_65N; Glyco_hydro_65m; Glyco_hydro_65C	<i>Candida_parapsilosis</i>	
PEX2_052250	PF00933.16	Glyco_hydro_3	<i>Ustilago_maydis</i>	
PEX2_054250	PF00331.15; PF00734.13	Glyco_hydro_10; CBM_1	<i>Magnaporthe_oryzae</i> (related: <i>Magnaporthe_grisea</i>)	
PEX2_056100	PF03659.9	Glyco_hydro_71		
PEX2_059920	PF03443.9	Glyco_hydro_61	<i>Gibberella_zeae</i> (related: <i>Fusarium_graminearum</i>)	
PEX2_067240	PF00933.16	Glyco_hydro_3		
PEX2_067560	PF00457.12	Glyco_hydro_11	<i>Botrytis_cinerea</i>	
PEX2_068000	PF00722.16	Glyco_hydro_16		

PEX2_069880	PF00295.12	Glyco_hydro_28	<i>Cochliobolus_carb onum</i>	
PEX2_070420	PF00704.23	Glyco_hydro_18	<i>Metarhizium_aniso pliae</i>	
PEX2_071120	PF00251.15; PF08244.7	Glyco_hydro_32N; Glyco_hydro_32C		
PEX2_072090	PF01670.11	Glyco_hydro_12	<i>Magnaporthe_oryz ae</i>	
PEX2_074460	PF03659.9	Glyco_hydro_71		
PEX2_075490	PF03663.9	Glyco_hydro_76		
PEX2_076310	PF07971.7	Glyco_hydro_92		
PEX2_076830	PF01301.14; PF10435.4; PF13363.1; PF13364.1	Glyco_hydro_35; BetaGal_dom2; BetaGal_dom3; BetaGal_dom4_5		
PEX2_080380	PF02837.13; PF00703.16	Glyco_hydro_2_N; Glyco_hydro_2		
PEX2_090750	PF00722.16	Glyco_hydro_16		
PEX2_093030	PF07335.6	Glyco_hydro_75	<i>Fusarium_solani</i>	
PEX2_094840	PF00295.12	Glyco_hydro_28	<i>Cochliobolus_carb onum</i>	24
PEX2_095490	PF00295.12; PF00465.14	Glyco_hydro_28; Fe-ADH	<i>Cochliobolus_carb onum</i>	
PEX2_097510	PF01055.21	Glyco_hydro_31	<i>Ustilago_maydis</i>	
PEX2_109740	PF03663.9	Glyco_hydro_76		
PEX2_110470	PF00295.12	Glyco_hydro_28	<i>Cochliobolus_carb onum</i>	48, 72
PEX2_038220	PF10282.4	Lactonase		24
PEX2_084130	PF10282.4	Lactonase		

PEX2_044440	GH18.hmm 8.20E-25 (dbCAN)	Glyco_hydro_18	<i>Metarhizium_anisopliae</i>	
PEX2_080610	GH16.hmm 1.80E-18 (dbCAN)	Glyco_hydro_16		
PEX2_104640	GH17.hmm 1.90E-21 (dbCAN)	Glyco_hydro_17	<i>Magnaporthe_oryzae</i>	
PEX2_013190	PF01095.14	Pectinesterase	<i>Botrytis_cinerea</i>	
PEX2_016450	PF00544.14	Pec_lyase_C	<i>Colletotrichum_gloeosporioides</i>	
PEX2_031470	PF00544.14	Pec_lyase_C	<i>Penicillium_digitatum</i>	
PEX2_107230	PF00544.14	Pec_lyase_C	<i>Penicillium_digitatum</i>	24, 48
PEX2_076520	PF12708.2	Pectate_lyase_3		
PEX2_079420	PF12708.2	Pectate_lyase_3		
PEX2_092020	PF12708.2	Pectate_lyase_3		24
PEX2_013030	PF14099.1	Polysacc_lyase		
PEX2_040240	PF07915.8	PRKCSH		
PEX2_087190	CE1 hmm. 6.30E-20 (dbCAN)	Putative esterase	<i>Gibberella_zeae</i> (related: <i>Fusarium_graminearum</i>)	

Proteolytic enzymes

PEX2_107240	PF01425.16	Amidase	<i>Candida_albicans</i>	
PEX2_006100	PF00026.18	Aspartic protease	<i>Leptosphaeria_maculans</i>	
PEX2_009280	PF00026.18	Aspartic protease	<i>Leptosphaeria_maculans</i>	72
PEX2_015270	PF00026.18	Aspartic protease	<i>Leptosphaeria_maculans</i>	
PEX2_085170	PF00026.18	Aspartic protease	<i>Candida_albicans</i>	

PEX2_089670	PF13933.1	HRXXH	<i>Blumeria graminis</i> <i>f. sp. hordei</i>	
PEX2_079330	PF00082.17	Peptidase_S8		
PEX2_028650	PF01828.12	Peptidase_A4		
PEX2_039390	PF01650.13	Peptidase_C13		24
PEX2_047070	PF00246.19	Peptidase_M14		
PEX2_037890	PF02129.13	Peptidase_S15		
PEX2_049380	PF00450.17	Peptidase_S10	<i>Ustilago maydis</i>	
PEX2_062610	PF04389.12	Peptidase_M28		
PEX2_084380	PF00450.17	Peptidase_S10	<i>Ustilago maydis</i>	
PEX2_087490	PF00450.17	Peptidase_S10	<i>Ustilago maydis</i>	
PEX2_089620	PF00450.17	Peptidase_S10	<i>Ustilago maydis</i>	
PEX2_106910	PF05577.7	Peptidase_S28		24, 48,72
PEX2_027670	PF05922.11; PF00082.17	Inhibitor_I9; Peptidase_S8	<i>Magnaporthe oryzae</i> (related: <i>Magnaporthe grisea</i>)	48, 72

Miscellaneous hydrolytic enzymes

PEX2_041030	PF00775.16	Dioxygenase_C		
PEX2_013750	PF07519.6	Tannase		
PEX2_075320	PF07519.6	Tannase		
PEX2_022440	PF00775.16	Dioxygenase_C		
PEX2_019000	PF01764.20	Lipase_3	<i>Gibberella zeae</i> (related: <i>Fusarium graminearum</i>)	
PEX2_099670	PF01735.13	PLA2_B	<i>Candida albicans</i>	
PEX2_010420	PF00149.23	calcineurin-like phosphoesterases		
PEX2_012250	PF01083.17	Cutinase		

(PHI data: white - not available, grey – no effect on pathogenicity, yellow - mixed output, light green – reduced pathogenicity, dark green - loss of pathogenicity; purple – lethal)

Table 2: Fungal and yeast vacuolar serine protease showing high homology to PePRT

Species		accession #	Annotation	Function characterization	% Identity to PePRT
<i>Penicillium</i>	<i>P. italicum</i>	KGO74337.1	proteinase inhibitor I9	none	97
	<i>P. camemberti</i>	CRL25709.1	peptidase S8/S53	none	96
	<i>P. digitatum Pd1</i>	XP_014533964.1	vacuolar serine protease	none	94
	<i>P. chrysogenum</i>	AAG44693.2	vacuolar serine protease	none	93
	<i>P. oxalicum</i>	AAG44478.1	vacuolar serine protease	none	79
<i>Aspergillus</i>	<i>A. fumigatus</i>	CAA73782.1	cellular serine proteinase	Reichard et al. 2000	76
<i>Cladosporium</i>	<i>C. herbarum</i>	AAX14379.1	vacuolar serine protease	none	67
<i>Verticillium</i>	<i>V. dahliae</i>	AGC74272.1	cerevisin	He et al. 2015	67
<i>Magnaporthe</i>	<i>M. oryzae</i>	EHA49897.1	subtilisin-like proteinase	Saitoh et al. 2009	65
<i>Saccharomyces</i>	<i>S. cerevisiae</i>	GAX70862.1	proteinase B	Moehle et al. 1987 Moehle et al. 1988 Moehle et al. 1989 Hirsch et al. 1992 Jones and Murdock 1994	53