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A Proteomic Analysis of Ripening Tomato Fruit Infected by *Botrytis cinerea*

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Abstract

Botrytis cinerea, a model necrotrophic fungal pathogen that causes gray mold as it infects different organs on more than 200 plant species, is a significant contributor to post-harvest rot in fresh fruit and vegetables, including tomatoes. By describing host and pathogen proteomes simultaneously in infected tissues, the plant proteins that provide resistance and allow susceptibility and the pathogen proteins that promote colonization and facilitate quiescence can be identified. This study characterizes fruit and fungal proteins solubilized in the *B. cinerea*-tomato interaction using shotgun proteomics. Mature green, red ripe wild type and *ripening inhibited (rin)* mutant tomato fruit were infected with *B. cinerea* B05.10 and the fruit and fungal proteomes were identified concurrently 3 days post-infection. One hundred and eighty-six tomato proteins were identified in common among red ripe and red ripe-equivalent *ripening inhibited* (*rin*) mutant tomato fruit infected by *B. cinerea*. However, the limited infections by *B. cinerea* of mature green wild type fruit resulted in 25 and 33% fewer defense-related tomato proteins than in red and *rin* fruit, respectively. In contrast, the ripening stage of genotype of the fruit infected, did not affected the secreted proteomes of *B. cinerea*. The composition of the collected proteins populations and the putative functions of the identified proteins argue for their role in plant-pathogen interactions.

Graphical abstract

Table of Contents (TOC) Graphic & Synopsis: Characterization of fruit and fungal proteins solubilized into the microenvironment of the interaction sites of *B. cinerea* infection of tomato fruit using shotgun proteomics. This proteomic approach obtains sufficient information to identify both pathogen and host proteins from sites of infection and to describe the diverse classes of proteins present

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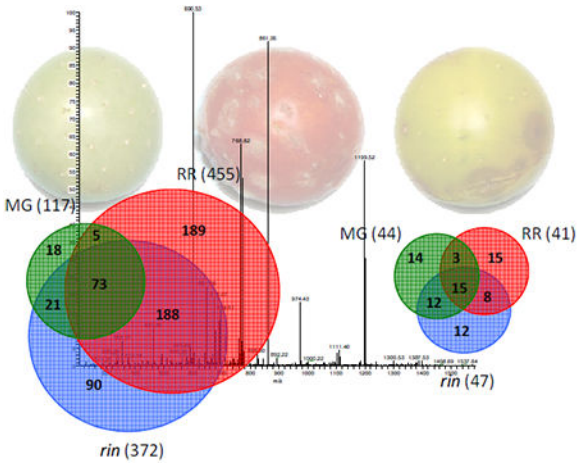
Supporting Information **Available**: This material is available free of charge via the Internet at http://pubs.acs.org



Keywords

Botrytis cinerea; tomato; host-pathogen interaction; mass spectrometry

Introduction

Botrytis cinerea (Pers.) [tel. *Botryotinia fuckeliana*] is a necrotrophic phytopathogenic fungus that grows particularly aggressively on the senescing aerial tissues of more than 200 plant species. *B. cinerea* causes gray mold on many economically important crops; pre- and post-harvest rotting infections by *B. cinerea* of fruits, vegetables, ornamental leaves and flowers cause significant losses.[1] Because *B. cinerea* infects many types of tissues under a variety of conditions, the fungus is likely to have diverse infection and growth strategies.[2] Furthermore, susceptibility to *B. cinerea* changes as the tissues develop and age. Green unripe fruit are largely resistant to rotting by *B. cinerea,* but ripe fruit are particularly susceptible, although *B. cinerea* is able to infect at least some non-ripening fruit.[3-5]

The interaction between plants and fungi includes communication that is undoubtedly in the form of proteins present in the microenvironment where the infections occur. The host-pathogen interaction is complex and dynamic, and is only partially elucidated by examining transcript abundances. Identifying which plant and which pathogenic proteins are present in infected sites may reveal some of the proteins that comprise the communication between the plant and the pathogen and may contribute to developing novel disease control strategies.

Limited proteomic information is available about plants interacting with pathogens. Proteomic work has cataloged plant, but not pathogen, proteomes in infected tissues and resulted in the identification of less than 100 proteins. A study of infections of pea with powdery mildew (*Erisphye pisi*) identified fewer than 100 pea proteins.[6] Infection of Arabidopsis suspension cells with *Pseudomonas syringae* resulted in the secretion collection of 45 Arabidopsis secreted proteins,[7] and similar sized collections of proteins were obtained from *Xanthamonas campestris* infection of *Brassica oleracea* although in this case most of the proteins identified were of bacterial origin.[8] The proteome of tomato fruits (*Lycopersicon esculentum*) infected with TMV identified 16 proteins. including several

pathogenesis-related (PR) proteins and antioxidant enzymes found that may be part of the plant resistance response to viral infection.[9] In another Arabidopsis proteome study, it was reported that 62 apoplastic Arabidopsis proteins were identified after 2D gel electrophoresis of Arabidopsis treated with oligogalacturonides.[10] Pathogen proteomics has focused on the on pathogen proteins obtained from cultures of fungal pathogens grown on synthetic and plant-derived sources such as glucose, cellulose, starch, pectin and partially purified total tomato fruit cell walls.[11-16]

In this study, we employed proteomic analysis of proteins released into the microenvironment of the infection sites of green and red tomato fruit with *B. cinerea* to identify the proteins produced by mature green (MG) and red ripe (RR) fruit in response to infection as well as proteins released by *B. cinerea*. We have identified proteins when only limited fungal growth is observed on MG fruit and when aggressive colonization is observed on ripened wildtype fruit and on non-ripening but susceptible *ripening inhibited (rin)* mutant fruit at the stage equivalent to ripe non-mutant fruit. Unlike previous studies of proteins secreted by *B. cinerea* which identified fungal proteins produced in cultures, this is the first analysis of the protein populations of both organisms simultaneously in a plant-fungal pathogen interaction.

Experimental Procedures

Fungal cultures

Botrytis cinerea (strain B05.10) conidia provided by Jan Van Kan (Wageningen University) were collected from sporulating cultures of the fungus grown on potato dextrose agar (Difco, Sparks, Maryland).

Inoculation

Mature green (MG, 34 days post anthesis) and red ripe (RR, 41 days post anthesis) tomato (*Lycopersicon solanum* cv. Ailsa Craig) fruit were harvested from plants grown in greenhouses in Davis, California. Fruit from plants with the *ripening inhibited* (*rin*) mutation in the Ailsa Craig background (provided by the Tomato Genome Resource Center, University of California, Davis) were grown at the same time in greenhouses and harvested at the RR-equivalent ripening stage (e.g. 41 days post anthesis). Six to ten fruit at each ripening stage were identified and collected from 3 to 4 plants of each genotype, sterilized by immersion in 10% bleach for 5 minutes followed by four rinses with distilled water.

Fruit inoculation was performed as described in Cantu et al. (2008).[3] All fruit were inoculated on the day of harvest to minimize variation due to storage and handling. At the time of inoculation, the fruit were punctured at sites that were 2 mm deep and 1 mm in diameter and 5-6 mm apart covering the stylar hemisphere of the fruit; 25-50 wound sites, depending on the size of the fruit, were made per fruit. Ten microliters of an aqueous suspension containing 5000 conidia of *B. cinerea* were placed in each wound site of the 4-5 fruit at each ripening stage of each genotype and were identified as the infected samples. Negative control material was wounded and 10 μl of H_2O was placed in the wound sites of similar numbers of fruit at the same ripening stages and genotypes from the same harvests

used for the infected samples. Fruit were incubated at 20°C in high humidity chambers. Three days after infection by *B. cinerea* were visibly apparent on the surface of the infected RR AC and ripe equivalent *rin* fruit, but infections were not observed on the MG AC fruit (Figure 1). The infected fruit had no visible evidence of contaminating infections and the wounded fruit had no evidence of microbial infections. The 4-5 fruit from a particular ripening stage (MG or RR), genotype (AC or *rin*) and treatment (infected or wounded) were combined. Six collections of extracted proteins were, therefore, evaluated: Infected MG AC (MG), infected RR AC (RR), infected RR-equivalent *rin* (*rin*), wounded MG AC, wounded RR AC and wounded RR-equivalent *rin*. Wounded (negative control) samples were analyzed by LC-MS/MS and we were not able to identify any proteins with statistically significant scores in these samples. The fruit (ca. 200 gm total weight) from each ripening stage, genotype and treatment were placed in 500 ml of protein extraction buffer (50 mM HEPES, pH 7.4, containing 2 mM DTT, 3 mM $NaHSO_3$, 2mM EDTA, 1.5 M NaCl) and gently shaken for 16 h at 4°C. The supernatants, after overnight extraction, were filtered through 2 layers of cheesecloth and then passed through 0.2 mm NYL membranes to remove all particulate material. The extraction buffer was dried at reduced pressure before the proteins in the extraction buffer were analyzed.

Isolation and Separation of Secreted Proteins by 1D-SDS –PAGE

Each dried extractant was resuspended in 20 mL of deionized water and dialyzed against deionized water using a Spectra/Por CE 1000 MWCO dialysis tubing (Spectrum Labs, Rancho Dominguez, CA). The desalted protein solution was lyophylized and resuspended in 2 mL of deionized water. Approximately equal amounts of total protein based on BCA protein assay, were separated by 1D-PAGE. Protein solutions from MG, RR and *rin* tomato fruit infected with *B. cinerea* was mixed with Laemmli sample buffer (Invitrogen, Carlsbad, CA). The proteins were separated on a 4-12% polyacrylamide precast gradient gel (Invitrogen, Carlsbad, CA) at 150 Volts for 1.5 h. The gels were silver stained to visualize the protein bands,[13] gels for trypsin digestion were not silver stained. All gels were done in duplicate; the duplicates were processed and analyzed separately as technical replicates.

In Gel Digestion

Gel lanes were divided into 5 sections of equal. Gel bands were then cut into smaller pieces (1×1 mm^2), dried in a vacuum centrifuge, and the proteins reduced by submerging the gel pieces in a 100 mM ammonium bicarbonate solution containing 10 mM dithiotheritol for 1 h at 55°C. The dithiothreitol solution was then replaced by the same volume of a 55 mM iodoacetamide solution containing 100 mM ammonium bicarbonate and incubated for 45 min in the dark. After alkylation, the gel pieces were treated with 100 mM ammonium bicarbonate and acetonitrile sequentially and then dried under vacuum centrifugation. The dried gel pieces were submerged in a solution containing 2 mg of trypsin in 100 mM ammonium bicarbonate and digestion of the proteins was carried out at 37°C overnight. Peptides were eluted from the gel fragments by collecting five sequential washings of the gel fragments; the gel fragments were washed once with ammonium bicarbonate followed by acetonitrile, and twice with 5% formic acid followed by acetonitrile. The eluted peptides in the collected washings were dried and resuspended in a 0.1% formic acid solution for mass spectrometric analysis.

LC-MS/MS Analysis

Trypsin digested secreted proteins were analyzed in duplicate for each of the tomato fruit samples and analyzed in separate mass spectrometry runs. An Agilent 1100 capillary LC (Palo Alto, CA) was attached with a T splitter to deliver ηL flow rates into the mass spectrometer. Five μm diameter C18 beads (Rainin, Woburn, MA) were packed into a pulled fused silica capillary (10.5 cm × 100 μm ID) under 1000 psi pressure using nitrogen gas. Peptide samples were loaded onto the column for 45 min under the same pressure. The loaded column was then washed with 95% buffer A for 10 min prior to being interfaced with the mass spectrometer.

Mobile phase A was 0.1% formic acid in water, and phase B was 99.9% acetonitrile/0.1% formic acid. Peptides were eluted from the column with a 90 min linear gradient from 5 to 60% buffer B at a flow rate of ∼200 ηL/min and injected directly into a LTQ linear ion trap mass spectrometer through an electrospray source (Thermo Fisher, San Jose, CA) using a voltage of 2500 V.

The instrument was set to acquire MS/MS spectra on the 9 most abundant precursor ions from each MS scan with a repeat count set of 3 and a repeat duration of 5 sec. Dynamic exclusion was enabled for 160 sec. Raw tandem mass spectra were converted into a peak list using ReAdW followed by mzMXL2Other algorithms. The peak lists were then searched using Mascot 1.9 (Matrix Science, Boston, MA).

Database Searching and Protein Identification

A target database was created by combining the *B. cinerea* BO5.10 from Broad Institute, MA (downloaded on 7/28/2009) and T4 databases from Genoscope, France (http://urgi.versailles.inra.fr/Species/Botrytis/Download; downloaded on 7/28/2009) with a tomato protein database from SOL Genomics Network, Cornell University, NY (ftp://ftp.sgn.cornell.edu/proteins/tomato_protein_with_hits.fasta) (released 7/5/2007). Combined databases are available for download as Supplemental Information Database).

A decoy database was constructed by reversing the sequences in the target database. Searches were performed against the target and decoy databases using the following parameters: (1) tryptic enzymatic cleavage with two possible missed cleavages; (2) peptide tolerance of 800 ppm; (3) fragment ion tolerance of 0.8 Da; and (4) variable modifications due to carboxyamidomethylation of cysteine residues (+ 57 Da) and deamidation of asparagine residues (+1 Da). Statistically significant proteins were determined for all of the samples at a 1% protein FDR using the ProValT algorithm as deployed in ProteoIQ (BIOINQUIRE, Athens, GA BioInquire is now NuSep, Bogart, GA).

Protein Functional Annotation

GOSlim terms were extracted from the AgBase web site (http://agbase.msstate.edu/cgi-bin/tools/GOanna.cgi)[17] using *GOSlim Viewer* after summarizing GO data. For Botrytis proteins with no assigned function homology searches were performed using the BlastP program against all nonredundant protein sequences in the NCBI database released on September 2009 (http://blast.ncbi.nlm.nih.gov/). Protein alignments were considered

significant if they were below an e-value threshold (≤ -50). Signal peptides in the deduced amino acid sequences were searched for using the SignalP web site.[18] Annotation based on association rules between CAZY families and the pfam domains was done with the CAZYmes Analysis Toolkit (CAT) from the BESC KnowledgeBase website (http://bobcat.ornl.gov/besc/index.jsp).

Results and Discussion

This study was designed to provide a qualitative global proteomic analysis. To this end, a simple, non-destructive buffer extraction method was adapted to collect both tomato fruit proteins as well as *B. cinerea* proteins released from the fruit and by the pathogen as a consequence of this host-pathogen interaction. A shotgun LC-MS/MS approach was used to identify tryptic peptide fragments of the collected proteins and provide researchers with access to a descriptive proteomic analysis. Data were obtained that identified proteins constituting the proteomes of tomato and *B. cinerea* three days after introduction of ungerminated spores into small wound sites on the fruit epidermis, a time point at which very little fungal growth is visible on the MG fruit but robust fungal growth is visible on the RR and the ripe equivalent *rin* fruit (Figure 2). Due to differences in fruit cell wall degradation because of the extent of fungal growth following infections of MG, RR and *rin* fruit[5] the greatest amount of tissue maceration occurs in infections of RR fruit. Less tissue maceration is observed in infections of ripe equivalent *rin* fruit and little maceration is seen in infections of MG fruit. Hence, the total amount of protein collected was significantly different from each type of infected fruit. A qualitative study was undertaken rather than attempting a semiquantitative or quantitative study due to the lack of information about the number of proteins involved in plant pathogen interactions, disparities in the amount of protein released in various infection conditions and the resulting impact of the abundance of particular proteins in the proteome pools.

Overview of the Proteomic Analysis of Tomato Fruit Infected with *B. cinerea*

A total of 588 tomato proteins and 79 *B. cinerea* proteins were identified in the present study. Venn diagrams (Figure 3) depicts the number of proteins from tomato (Figure 3a) and *B. cinerea* (Figure 3b) that were found in common in the pairings as well as those which were unique to material from infected MG and RR wild type or *rin* mutant fruit. We identified 119, 456 and 374 tomato proteins from infected MG, RR and *rin* tomato fruit, respectively. Only 13% of the tomato proteins (74 tomato proteins) were in common among all collections, suggesting that while infections of unripe or ripe fruit released a common set of proteins, most of the proteins were unique and determined by the ripening stage or genotype of the host fruit tissue. In contrast, similar numbers of *B. cinerea* proteins were identified in all of the collections; 44, 41 and 47 fungal proteins were identified from infections of MG, RR and *rin,* respectively. Of these, 15 proteins (~34%) of the *B. cinerea* proteins in each collection were common among all the collections. Supplemental Information Table 1 summarizes the spectra and proteins in each of the collections that were identified as tomato proteins and the number that were identified as *B. cinerea* proteins. The tomato proteins identified in MG, RR and *rin* fruit comprised 73, 92 and 89% respectively of

the total proteins identified, and *B. cinerea* proteins comprised the remainder. Spectral analysis reveals an essentially identical profile.

To obtain an overview of how proteins from the tomato-*Botrytis* interaction are associated with various molecular functions, Gene Ontology (GO) Slim terms were used to categorize the proteomes in the interaction. The functional assignments of the identified tomato and *Botrytis* proteins with molecular functions and biological processes in GOSlim terms based on the AgBase database are shown in Supplemental Information Figures 1-4.

Tomato Proteins

A total of 588 tomato proteins were identified from the proteins collected from Botrytis infections of MG and RR wild type and *rin* fruit (Supplemental Information Table 2 and 3). We focused on tomato defense related proteins for a more detailed analysis. Plants have evolved constitutive and inducible protective mechanisms of antimicrobial defense[19] known as the defense or pathogen responses. Defense-related proteins[20] are synthesized in response to biotic stress. Genes encoding pathogenesis-related proteins (PR proteins) are specifically induced in pathological or related situations and 17 families have been identified.[21] Generally, these genes have been studied by transcript accumulation, which may or may not correlate with actual protein levels. The proteins identified in this work with putative biological or biochemical functions similar to the 17 PR protein families were grouped together and included as defense related proteins along with peroxidases, proteases and protease inhibiting proteins. The 114 defense related proteins were further classified into four sub-categories: PR proteins (43 proteins), proteases (43 proteins), peroxidases (13 proteins) and protease inhibiting proteins (15 proteins).

Among the 43 PR Proteins observed (Table 1), 12 chitinases were identified. Chitinases (EC 3.2.1.14) catalyze the hydrolysis of chitin, a linear homopolymer of β-1,4-linked N-acetylglucosamine (GlcNAc) residues. Chitinases constitute the second largest group of antifungal proteins,[22] and a variety of chitinases have been identified during other plant-microbe interactions.[9, 23-26] In addition, 10 1,3- and 1,4-β-endoglucanases were identified. The antifungal activity of these plant endoglucanases is thought to be a result of their hydrolysis of the structural 1,3-β-glucan present in the fungal cell wall, particularly at the hyphal apex of filamentous molds where glucan is most exposed, leading to cell lysis and cell death.[27] Chitinases and β-1,3-glucanases are well-characterized classes of PR proteins and together can effectively inhibit fungal growth.[28-30] Plant inhibitors of polysaccharide hydrolases produced by pathogens have been shown to provide reduced susceptibility to infections.[4] A xyloglucan-specific fungal endoglucanase inhibitor protein[31] was frequently detected in all protein collections and a polyglacturonase inhibiting protein (PGIP) whose expression has been shown to reduce susceptibility of tomato fruit to *B. cinerea*[4] was identified less frequently but in all collections. Other proteins in the PR category include a disease response protein, an elicitor-inducible protein, a glycosyl hydrolase, three osmotin like proteins, and thaumatin.

Forty three proteases were a second subcategory of defense related proteins. Plants have a large arsenal of proteolytic enzymes that regulates the fate of proteins. These proteins are proposed to have primarily a housekeeping role. However, proteases also play a key role in

the regulation of biological processes, such as the recognition of pathogens and pests and the induction of effective defense responses.[32-34]

Thirteen peroxidases were identified among the 114 defense related proteins. In the course of pathogen–plant interaction, peroxidases are key enzymes in the detoxification systems involved in scavenging reactive oxygen forms, whose increased generation is closely associated with the induction of plant defense reactions.[35] In tomato it has been shown that heat treatment after inoculation induces peroxidases that prevent *B. cinerea* development.[36]

We identified 15 protease inhibiting (pin) proteins in the 114 defense proteins and, more than 60% belong to the Type I class of pin proteins. Pin proteins are among the defense proteins in plant tissues that are both developmentally regulated and induced in response to insect and pathogen attack.[37] Type I pin proteins are best known in potatoes but are found in other plant species, for example, barley endosperm chymotrypsin inhibitor is a Type I pin.[38] It has been shown that protease inhibitors isolated from healthy bean and tomato plants reduced the activities of proteases from *Fusarium solani* and *C. lindemuthianum*.[39, 40] In tomatoes, serine protease inhibitors I and II accumulate in endosperm cell walls and in secretory cells of root cap, and are secreted into the milieu.[41]

Fruit cell wall related proteins—Forty-four proteins from the three collections were assigned to the cell wall related categories (Table 2). Fruit cell wall related proteins include subcategories of cell wall (structural) proteins and proteins thought to cause cell wall modifications (both catabolic and anabolic).

Other tomato proteins—The other categories of tomato proteins identified in the collections include sub-classes that have not been implicated directly in host-pathogen interaction. However, such a role for these proteins cannot be disregarded *a priori*. The presence of these proteins is most likely due to cell lysis during the infection, thus they should be considered an indirect response to the presence of the pathogen.

Carbohydrate-active enzymes from tomato—Families of structurally-related catalytic and carbohydrate-binding modules (CBMs) for 93 tomato proteins (Supplemental Information Table 2, Supplemental Information Figure 5) were assigned based on CAZYmes Analysis Toolkit (CAT) (http://bobcat.ornl.gov/besc/index.jsp); this represents 16% of the 586 tomato proteins identified. One major class of CAZy identified for tomato proteins was the glycoside hydrolases (GH) with 45 members. CBM, carbohydrate esterase (CE) and glucosyl transferase (GT) families had 24, 18 and 6 members, respectively.

Tomato transcriptome and proteome analysis—To gain insights into the accumulation of specific proteins, we compared the tomato protein data with microarray transcriptome data from Cantu *et al* (2009) [5]. The changes in expression from the transcriptome analysis that were also observed in the proteome analysis are listed in Supplemental Information Table 4. Of the 186 identified tomato proteins, 32 had transcript probes represented on the microarray.

In general, there was no significant up or down regulation of any transcripts when MG or RR fruit were inoculated or wounded; transcript abundance of these 32 genes stayed remarkably constant, in both ripening stages and in healthy, wounded or infected tissues. Transcriptional changes observed in both MG and RR fruit in response to *B. cinerea* suggested pathways transcriptionally activated by *B. cinerea* regardless of the ripening stage of the fruit. In most tomato-botrytis combinations expression of an embryo-abundant protein-related, a saposin B domain-containing protein, a putative pectinesterase, and two FLA1 (Fasciclin-Like Arabinogalactan 1) transcripts was activated by the fungus and proteins from these genes also accumulated.

In infected *rin* fruit only a copper ion binding / oxidoreductase (SGN-U314232; BG735509) was identified from both transcriptome and proteome analysis. Fourteen probe sets were identified in proteins unique to RR fruit and 3 out of 14 are involved in defense mechanisms (SGN-U317362, SGN-U317362 and SGN-U316817). Between RR and *rin* fruit, nine probe sets were linked to 9 proteins. Three out of the 9 are cell wall defense related proteins (SGN-U318232, SGN-U318232 and SGN-U318232) and 2 proteins were involved in defense mechanisms (SGN-U315473 and SGN-U315473). Four probe sets matched two proteins found in common in MG, RR and *rin* fruit (SGN-U320398 and SGN-U322781). Overall, no close correlation was found comparing results of the proteome and transcriptome. The low correlations between the presence of a transcript and the presence of its corresponding protein suggest that post-transcriptional regulatory mechanisms play an important role in the fruit-pathogen interaction and has not been described previously. Osorio et al., (2011) reported also a low correlation between transcriptome and proteome data, especially in MG fruit.[42]

Botrytis cinerea Proteins

Seventy-nine proteins encoded by *B. cinerea* genes during the infection of tomato fruit were identified in the collections from MG, RR and *rin* infected tomatoes (Table 3, Supplemental Information Figure 6). In order to identify secretory signal peptides in *B. cinerea* proteins, we used the SignalP 3.0 algorithm (Table 3). Using this algorithm, 58 out of 79 fungal proteins were found to have a secretory signal peptide, representing 73% of the total fungal proteins, while the remaining 21 (27%) proteins did not contain an identifiable signal peptide. However, use of the WoLF PSORT algorithm (http://wolfpsort.seq.cbrc.jp) indicated an additional seven proteins out of the remaining 21 were categorized as extracellular because of possible signal peptides. Four of the proteins were found to be intracellular when *B. cinerea* was grown in 1% carboxymethylcellulose,[11] raising the possibility that the presence of these proteins in our study is due to lysis of *B. cinerea* cells. Further studies will determine whether these proteins are truly secreted or are present as the result of either autolysis or lysis occurring as a host's defense response to the pathogen.

Plant cell wall degrading enzymes—To accomplish successful penetration and colonization of a plant, pathogenic fungi must break the cell wall. Degradation of plant cell wall compounds by plant pathogens is a complex process involving the synergistic action of a large number of extracellular cell wall degrading enzymes and proteins that help pathogens penetrate and colonize plant tissues. Enzymatic cleavage of the plant cell wall releases

carbohydrates, which can become a carbon source for the pathogen. Of the 79 *B. cinerea* proteins detected, 36 (46%) were classified as cell wall degrading proteins are listed in Table 4. BofuT4_P131540.1 with homology to a PME-1 was identified in MG, RR and *rin* tomato. Other PMEs identified included BC1G_00617.1 in MG and *rin* tomato and BC1G_11144.1 in MG tomato. Interruption of *Bcpme1* reduces pathogenicity on several plant hosts,[42] indicating that pectin methyl esterase contributes to *B. cinerea* pathogenicity.

Endo-PGs were also identified in the present study, including some endo-PGs that had been previously cloned and identified in the *B. cinerea* genomes.[43] Endo-PG activity is regulated by at least six endo-PG genes, and *B. cinerea* mutants of endo-BcPG1 and endo-BcPG2 have reduced virulence on tomato and other hosts.[44, 45] Peptides from BcPG1, BcPG2 and an exo-PG were identified in the present study. Although this study was designed to provide a qualitative global proteomic analysis, some proteins showed high spectral counts and we consider that is important to mention them because they are likely to be particularly abundant. For example, endo-BcPG1 (BC1G_11143.1) was the most abundantly detected *B. cinerea* protein in all collections (89, 126 and 305 spectra) from infections of MG, RR and *rin* tomatoes, respectively; while BcPG2 was identified in the *rin* mutant with three spectral counts and exo-PG was identified in MG tomato, also with three spectral counts. The expression of exo-PGs has been previously identified in cucumber leaves inoculated with spores of *B. cinerea*.[46] This expression either increased with time of culture (exo-PG I) or was transiently expressed soon after the start of culture (exo-PGII), suggesting that the exo-PGs play an important role in pathogenesis at an early stage of infection as well as in tissue maceration of host plants.[46]

β-Galactosidase (BC1G_03567.1) is another fungal enzyme identified in MG, RR and *rin* tomato. Plants also express β-galactosidases, which degrade plant cell wall pectins during cell wall loosening that occurs prior to cell elongation.[47] Presumably *B. cinerea* uses β-galactosidases to facilitate the breakdown of fruit cell walls, but its spectral abundance from infected fruit suggests that it may have a more significant role than had been previously assumed.

B. cinerea cellobiohydrolases were only identified in collections from MG and/or *rin* fruit but not in RR tomato fruit. In MG tomato fruit infected by *B. cinerea*, three putative cellobiohydrolase or cellulase proteins (BC1G_14702.1, BC1G_06035.1, and BC1G_10880.1), all with a putative cellobiohydrolase or cellulase function, were identified. In infected *rin* fruit, BC1G_14702.1, BCG_03188.1 and BC1G_10880.1 were also identified. Because transcripts of the *B. cinerea* cellobiohydrolase gene are absent in infected tomato leaves, it has been suggested that cellobiohydrolase does not play an important role in infection of leaves, but its role in infection of fruit is not known.[48] Cellulase enzyme activity from *Trichoderma reesie* produces a soluble inducer from insoluble cellulose,[49, 50] which triggers the expression of cellobiohydrolase in *T. reesie*.[51] *Claviceps purpurea* cellulolytic enzymes are expressed during the infection process of rye.[52] These findings suggest that pathogen cellulases could be involved in the penetration and degradation of host cell walls, thus playing a role in the infection process.

Lacasses are another of the classes of *B. cinerea* proteins identified, and are present in either all the collections (BC1G_08553.1) or in proteins from infected RR fruit only (BC1G_12487.1). During infection, Botrytis cleaves the polysaccharides within the host cell wall, releasing plant wall fragments that in some hosts may result in the production of phytoalexins, a class of compounds which act as antifungal agents involved in the host response to pathogen attack.[53, 54] Hydrogen peroxide is one of the forms of active oxygen species (AOS), which can arise from the response of plant tissues to infection by pathogens.[55] Therefore, it may not be unexpected that Botrytis would produce laccases which are capable of inactivating these compounds.

Five *B. cinerea* glucosidases were identified in the present study (BC1G_12859.1, BC1G_10221.1, BC1G_02364.1, BC1G_04151.1 and BC1G_11898.1). The importance of glucosidases was demonstrated when a positive correlation was found between the *β*-glucosidase activity of *B. cinerea* in liquid culture and pathogenicity, as expressed in the area of lesion caused by *B. cinerea* in different hosts.[56]

Pathogenicity-related proteins—The category of *B. cinerea* pathogenicity-related proteins identified contained a homologue of Snodprot (BC1G_02163.1) with a large number of spectral counts in all samples (Table 3) as also seen in previous studies (Table 4). The Botrytis Snodprot homologue (*BcSPL1*) has a similar sequence to cerato-platanin, and is induced by plant ethylene production at an early stage of infection, and has been reported as an elicitor of pathogen responses and mutants have reduced pathogenicity.[57] Elevated *BcSPL1* expression has been observed during the first 72 hours after infection. Tomato plants contain a saponin called α-tomatine that has been proposed to kill sensitive cells by binding to cell membranes resulting in leakage of cell components.[58] It has been shown that α-tomatine kills a broad range of fungi *in vitro* and functions as a resistance substance against phytopathogens in tomato.[59] As is expected, fungi that invade saponin-containing plants are likely to have a strategy to protect themselves from these saponins and *B. cinerea* appears not to be the exception, as it produces tomatinase, an enzyme in the GH10 family of β-glucosyl hydrolases that degrades α-tomatine.[60] In the NCBI protein database there are 75 tomatinase amino acid sequences.[61] A *B. cinerea* xylosyl hydrolase that degrades α-tomatine has also been purified,[61] but its relationship to other fungal glycosyl hydrolases is unclear as the protein has not been sequenced nor has the gene encoding the enzyme been identified.

Carbohydrate-active enzymes from Botrytis—Families of structurally-related catalytic and carbohydrate-binding modules for 28 *Botrytis* proteins (Table 3, Supplemental Information Figure 6) were assigned based on the CAZYmes Analysis Toolkit (CAT). One major class of CAZy was identified for Botrytis proteins: glycoside hydrolases (GH) with 21 members. Three minor groups, CBMs, CEs and polysaccharide lyases (PLs), were identified with 6, 3 and 1 members, respectively.

Common Botrytis proteins—Fifteen proteins from Botrytis were identified in common in MG, RR and *rin* infected tomato (Table 4). Of these, 12 Botrytis proteins had been reported in at least one other secretome study[12-15] and 3 were identified as unique to this study. A few proteins (BofuT4_P108020.1, BC1G_14129.1, BC1G_12374.1 and BCIG_00896.1) appear worthy of special comment.

BofuT4_P108020 has homology to a nucleoside diphosphate kinase (NDPK) which has been identified in other wound-response studies.[62] In addition, it has been shown that NDK in *Neurospora crassa* together with a catalase plays an important role in supporting the survival of conidia under oxidative and light-induced stress including singlet oxygen.[63]

Three proteins are present in all samples and their function could not be identified after a Blastp analysis. BC1G_12374.1 and BCIG_00896.1 were previously identified in other secretome studies, and BC1G_14129.1 has not been reported previously. Their presence in all three studies (Table 4) suggests that these proteins could be involved in pathogenicity. It would be of interest to isolate the genes, over-express them, and characterize the resultant proteins to evaluate their role in pathogenicity.

Another feature interesting to highlight from these common proteins is their glycosylation. Many proteins can present with either *O*-linked or *N*-linked glycosylation or both (Table 4). Filamentous fungi are known to carry high-mannose *N*-glycans.[64] *O*-Mannosylation is found in glycoproteins of many higher eukaryotes as well as in most fungi,[65] including the filamentous fungi.[66] Cell wall degrading enzymes, especially PGs from a single strain of fungus, may exist in a variety of isoforms.[67-69] The PG isoforms may each exist as a series of glycoforms, and may vary in their mode of action as well as in their ability to interact with their plant target (i.e., specific pectin classes of PGIPs).[67, 70] In addition, the PGIPs of a single plant species may be present as a set of isoforms, each of which exists as a series of glycoforms.[71] Protein glycosylation has proven to be important in maintaining protein structure and function and can play a key role in protein-protein interactions.[72, 73] In the case of the PG-PGIP complex, this structural variation provides the potential for a wide range of specificity in their interactions within any plant–pathogen pairing. Whether glycosylation of proteins might alter the proteolytic stability of a given protein or its ability to interact with its plant target needs to be investigated, since it could be one of the critical factors in determining whether the fungus is a viable pathogen.

Conclusion

When pathogens encounter plant tissues, proteins from both the plant and the pathogen enable infections and form responses to the interaction. The proteins produced by the pathogen include those that facilitate the pathogen's penetration and growth on the plant tissue, those that repress resistance responses by the plant, and those that allow the pathogen to utilize the nutrient resources within the plant. The proteins produced by the plant include those that limit pathogenic infection, those that facilitate growth of the infecting organism, and those that protect the plant tissue from additional damage. By documenting simultaneously the proteomes of the pathogen and host in the microenvironment of infection sites, the plant proteins that provide resistance and facilitate susceptibility and the pathogen proteins that promote colonization and quiescence can be identified.

In order to grow, a plant pathogenic fungus must secure a carbon source from the plant. A complex cocktail of extracellular enzymes is secreted from the pathogen to fragment polymers, such as cellulose, lignin, proteins and lipids, and then deliver the resulting simple sugars, amino acids, and fatty acids to fungal hyphae, allowing the pathogen to develop and

fulfill its life cycle. In response, the host will produce a battery of defense mechanisms to protect itself from the intruder (Figure 4).

The proteome analysis described in this work allowed simultaneous identification of *B. cinerea* and tomato fruit proteins released into the microenvironment of fruit tissue infected by *B. cinerea*. These results show that this approach is feasible, both for obtaining sufficient information to identify both pathogen and host proteins from sites of infection and to describe the diverse classes of proteins. The results obtained show concurrent information about the host and the pathogen proteomes, identifies significant number of diverse proteins involved in pathogenicity and proteins involved in protection against the oxidative stress response by the host. The identification of proteins with unknown function opens new research avenues, possibly identifying new biological functions that may play important roles in the plant-pathogen interaction.

The data obtained reveal that unsuccessful infections of green fruit result in the identification of 25 and 33% fewer defense related proteins encoded by the host plant as compared to infections of red and non-ripening *rin* tomato fruit, and that regardless of whether ripe red or non-ripening *rin* tomato fruit are successfully infected by *B. cinerea*, but differences in the plant proteomes could be due to the extent of tissue maceration or differences in response by the fruit. In contrast, irrespective of whether *B. cinerea* is able to infect MG, RR or *rin* tomato fruit, the proteins coming from the fungus in the infection microenvironment are similar. As noted above, however, shotgun studies cannot detect isoforms or post-translationally modified forms of the proteins, which may prove to be important factors in the change in host-pathogen interactions that lead to susceptibility *vs* resistance.

This proteome analysis identifies both plant and pathogen proteins with as yet unknown functions which may play a crucial role in plant-pathogen interactions. Proteomics analysis at different times during infection, along with the use of comparative and quantitative techniques, may allow identification of key proteins for recognition and early defense or attack during the interaction. Plant-pathogen proteomics is still in its early stage, but will likely become an active field with a large impact on plant biology. The rapid development of new proteomic analysis techniques is revolutionizing the study of plant-pathogen interactions and shedding more light on the complex network of signaling cascades involved in plant defense responses. Finally, a plant-pathogenic fungus proteome database would be of help to the scientific community studying either the pathogen or the host, and will assist in identifying those genes that are related to different infection and developmental stages as well as growth conditions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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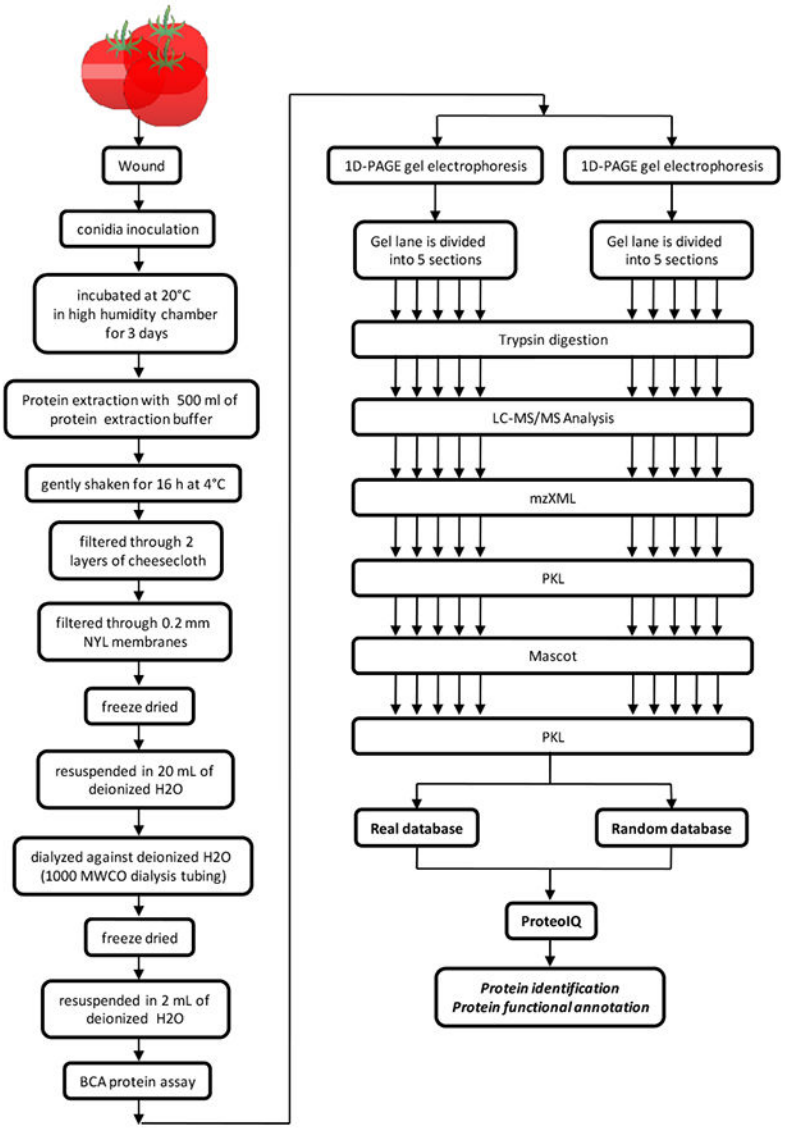

Figure 1.

Schematic depiction of protein extraction and peptide preparation for proteomic analysis for RR, *rin* and MG tomato fruit infected with *B. cinerea*

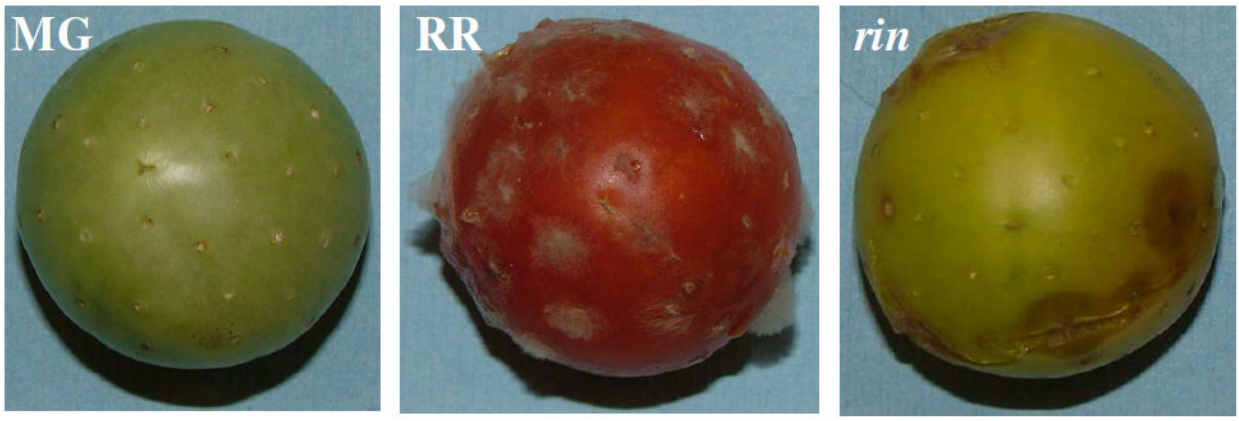

Figure 2.
Tomato fruit infected by *Botrytis cinerea* at the MG (31 dpa), RR (42 dpa) stages of wild type AC and at 42 dpa of the *rin* non-ripening mutant.

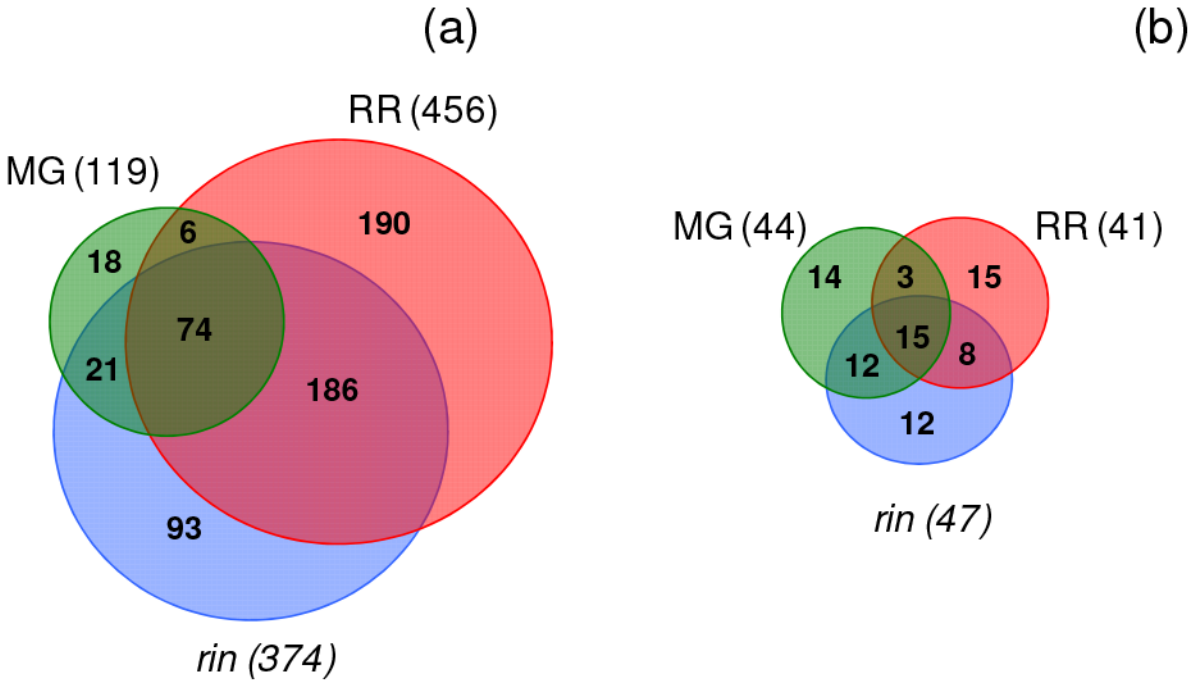

Figure 3.

Area-proportional Venn diagrams showing overlap of tomato proteins (a) and *Botrytis cinerea* proteins (b) identified from RR, MG and *rin* tomatoes infected with *Botrytis cinerea*.

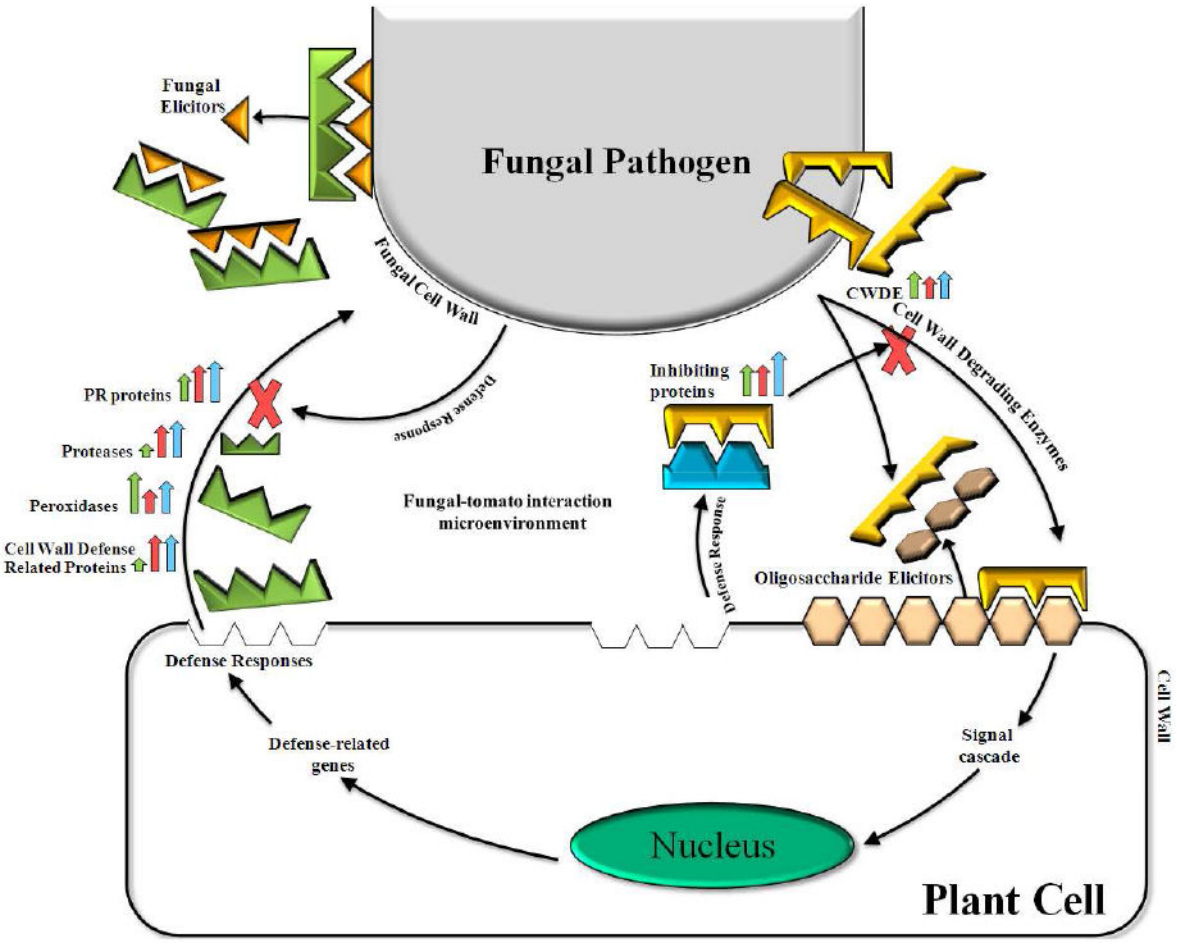

Figure 4.

Hypothetical model representing the proteins secreted during a plant-fungal interaction. *Pathogenic fungi facilitate their colonization of plant tissue by producing Cell Wall Degrading Enzymes (CWDEs) that fragment plant cell wall polysaccharides. The oligosaccharides that are generated by these glycanases provide the fungus with a carbon source but are also perceived by and elicit defense responses in the host plant. As part of that response, plants produce proteins that inhibit fungal glycanases and thereby increase the life time of the biologically active oligosaccharides. The fragmentation of the fungal cell wall by plant defense related proteins also generates oligosaccharides that induce plant defense responses. The fungus itself may in turn produce defensive proteins to prevent the degradation of its own cell wall and limit its perception by the plant. Thus, the interplay between fungal and plant glycanases and their respective inhibitors may in large part determine the outcome of attempted pathogenesis. The height of the colored arrows indicates the relative abundance of that particular protein group identified when MG (green arrows), RR (red arrows) and rin (blue arrows) tomatoes are infected with B. cinerea.*

Table 1

Identified tomato defense related proteins

Sub-category	Gene Id	Putative Function	SignalP	MG		RR		*rin*		CAZy Families
				Peptide	Spectra	Peptide	Spectra	Peptide	Spectra	
Pathogenesis related proteins	SGN-U312944	beta-1,3-Endoglucanase	0.999 Y	21	73	22	56	32	167	GH17
	SGN-U312558	Basic Endochitinase	0.001 N	20	68	21	66	27	110	GH19|CBM18
	SGN-U312562	Basic Endochitinase	0.113 N	17	74	23	110	33	195	GH19|CBM18
	SGN-U314071	Xyloglucan-specific fungal Endoglucanase inhibitor protein	0.998 Y	17	43	20	83	30	129	-
	SGN-U312653	Thaumatin	0.994 Y	17	44	15	52	14	39	-
	SGN-U342004	beta 1,3 glucanase	0.803 Y	17	29	12	14	13	24	GH17
	SGN-U314797	P14	1.000 Y	16	79	6	20	14	51	-
	SGN-U316008	P2	0.012 N	15	64	14	79	18	82	-
	SGN-U313265	Basic Endochitinase	0.000 N	14	33	8	17	18	52	GH19
	SGN-U312654	Osmotin like	0.656 Y	12	30	12	37	13	35	-
	SGN-U312559	Basic Endochitinase	0.966 Y	11	57	11	54	14	79	GH19|CBM18
	SGN-U312561	Basic Endochitinase	0.008 N	8	40	9	36	12	64	GH19|CBM18
	SGN-U312560	Basic Endochitinase	0.017 N	7	15	7	16	11	23	GH19|CBM18
	SGN-U312367	PR-1 precursor	0.999 Y	6	20	8	33	11	21	-
	SGN-U333664	Basic Endochitinase	0.000 N	6	10	5	8	8	17	GH19|CBM18
	SGN-U314382	beta 1,3 glucanase	0.001 N	6	10	2	4	3	7	GH17
	SGN-U313266	Basic Endochitinase	0.000 N	4	6	4	4	1	1	GH19
	SGN-U313763	PR protein	0.880 Y	4	14	3	8	3	11	CBM18
	SGN-U315737	PR protein	0.000 N	4	5	1	3	3	3	-
	SGN-U312368	PR protein	0.000 N	3	6	7	26	4	9	-
	SGN-U322252	Chitinase	0.001 N	3	7	3	3	4	6	GH18
	SGN-U316224	Chitinase	0.959 Y	3	4	3	4	0	0	GH19|CBM18
	SGN-U312323	Osmotin like protein	0.999 Y	2	3	0	0	0	0	-
	SGN-U315727	PGIP1	0.000 N	1	2	5	8	5	16	-
	SGN-U324260	Endo beta 1,4 glucanase	0.958 Y	1	1	1	2	3	5	GH9|GH19|CBM18
	SGN-U312369	PR protein	0.000 N	1	1	1	1	0	0	-

Sub-category	Gene Id	Putative Function	SignalP	MG		RR		*rin*		CAZy Families
				Peptide	Spectra	Peptide	Spectra	Peptide	Spectra	
	SGN-U312981	PR protein	0.004 N	0	0	13	215	10	87	-
	SGN-U317470	PR protein	0.000 N	0	0	6	46	3	4	-
	SGN-U317219	beta 1,3 glucanase	0.000 N	0	0	2	2	1	1	-
	SGN-U319531	Basic Endochitinase	0.998 Y	0	0	1	1	1	1	-
	SGN-U316555	beta 1,3 glucanase	0.004 N	0	0	1	1	0	0	GH17|CBM43
	SGN-U322253	Chitinase	0.985 Y	0	0	1	1	0	0	GH18
	SGN-U316817	Endo 1,3 beta glucosidase	0.998 Y	0	0	1	1	0	0	GH17|CBM43
	SGN-U345256	Endo-1,4-glucanase	0.651 Y	0	0	1	1	1	2	-
	SGN-U318558	Osmotin like protein	1.000 Y	0	0	1	1	1	3	-
	SGN-U319298	PR protein	0.000 N	0	0	1	1	2	2	-
	SGN-U317352	Disease resistance-responsive protein	0.991 Y	0	0	0	0	1	1	-
	SGN-U317182	Elicitor-inducible protein	0.020 N	0	0	0	0	1	1	-
	SGN-U315668	Endo 1,3 beta glucosidase	0.076 N	0	0	0	0	1	1	GH17
	SGN-U321793	Endo 1,3 beta glucosidase	0.000 N	0	0	0	0	1	1	GH17|CBM43
	SGN-U340225	Glycosyl hydrolase	0.000 N	0	0	0	0	1	1	GH17|CBM43
	SGN-U315428	PR protein	1.000 Y	0	0	0	0	2	4	-
	SGN-U330712	PR protein	0.000 N	0	0	0	0	2	2	-
Proteases	SGN-U313379	Aminopeptidase	0.001 N	0	0	6	7	2	2	-
	SGN-U317362	Aminopeptidase	0.009 N	0	0	1	1	0	0	-
	SGN-U312783	ATP-dependent protease	0.016 N	0	0	2	2	0	0	-
	SGN-U322932	Carboxypeptidase	0.000 N	0	0	3	3	0	0	-
	SGN-U322736	Nucellin	0.057 N	0	0	0	0	1	1	-
	SGN-U313835	Peptidase	0.000 N	11	23	15	42	15	37	-
	SGN-U334617	Peptidase	0.000 N	4	6	5	12	7	9	-
	SGN-U319568	Peptidase	0.999 Y	1	1	0	0	3	3	-
	SGN-U321102	Peptidase	0.000 N	0	0	2	2	3	3	-
	SGN-U324600	Peptidase	0.987 Y	1	1	0	0	2	3	-
	SGN-U316263	Peptidase	0.000 N	0	0	0	0	1	1	-
	SGN-U317724	Protease	0.000 N	3	5	7	9	23	43	-

Sub-category	Gene Id	Putative Function	SignalP	MG		RR		*rin*		CAZy Families
				Peptide	Spectra	Peptide	Spectra	Peptide	Spectra	
	SGN-U322187	Protease	0.002 N	2	4	14	29	14	33	-
	SGN-U314479	Protease	0.999 Y	2	3	6	9	15	30	-
	SGN-U318298	Protease	0.991 Y	1	1	4	5	11	16	-
	SGN-U313775	Protease	0.994 Y	1	2	0	0	6	10	-
	SGN-U315460	Protease	0.715 Y	0	0	6	11	8	13	-
	SGN-U312375	Protease	0.000 N	0	0	14	22	4	4	-
	SGN-U315473	Protease	0.001 N	0	0	7	12	7	9	-
	SGN-U312919	Protease	0.971 Y	0	0	8	11	4	4	-
	SGN-U316289	Protease	0.000 N	0	0	0	0	4	7	-
	SGN-U313378	Protease	0.001 N	0	0	9	10	3	3	-
	SGN-U313997	Protease	1.000 Y	3	4	13	39	3	8	-
	SGN-U315317	Protease	0.996 Y	0	0	2	2	2	4	-
	SGN-U313773	Protease	0.002 N	2	2	0	0	1	1	-
	SGN-U314619	Protease	0.995 Y	0	0	3	6	3	6	-
	SGN-U316051	Protease	0.000 N	0	0	7	9	2	2	-
	SGN-U322487	Protease	0.000 N	0	0	1	1	2	3	CBM5
	SGN-U315577	Protease	0.997 Y	0	0	2	4	2	4	-
	SGN-U320558	Protease	0.000 N	0	0	1	1	0	0	-
	SGN-U316057	Protease	0.650 Y	0	0	0	0	1	1	-
	SGN-U312592	Protease	0.999 Y	2	3	1	2	1	1	-
	SGN-U326813	Protease	0.791 Y	0	0	2	2	0	0	-
	SGN-U317291	Protease	0.002 N	0	0	1	1	0	0	-
	SGN-U337147	Protease	0.002 N	0	0	1	2	0	0	-
	SGN-U321739	Protease	0.008 N	1	1	0	0	0	0	-
	SGN-U312589	Protease	0.953 Y	2	2	0	0	4	5	-
	SGN-U312588	Protease	0.233 N	1	1	0	0	2	2	-
	SGN-U313739	Serine carboxypeptidase	0.999 Y	0	0	17	27	5	6	-
	SGN-U323007	Serine carboxypeptidase	0.000 N	0	0	6	9	2	2	-
	SGN-U316728	Serine carboxypeptidase	0.098 N	0	0	1	2	1	1	-
	SGN-U322415	Serine carboxypeptidase	0.987 Y	0	0 0	2	2	0	00	-

Sub-category	Gene Id	Putative Function	SignalP	MG		RR		*rin*		CAZy Families
				Peptide	Spectra	Peptide	Spectra	Peptide	Spectra	
	SGN-U315356	Ubiquitin specific protease	0.000 N	0	0	1	1	0	0	-
Peroxidases	SGN-U315418	Peroxidase	0.000 N	7	18	1	1	12	26	-
	SGN-U313411	Peroxidase	0.997 Y	9	26	10	18	11	27	-
	SGN-U321125	Peroxidase	0.000 N	7	11	0	0	4	4	-
	SGN-U321126	Peroxidase	0.032 N	8	16	0	0	4	6	-
	SGN-U323529	Peroxidase	0.000 N	18	61	2	2	3	4	-
	SGN-U315693	Peroxidase	0.957 Y	4	20	3	12	3	6	-
	SGN-U316807	Peroxidase	0.989 Y	5	12	3	3	2	6	-
	SGN-U315420	Peroxidase	0.957 Y	0	0	0	0	1	1	-
	SGN-U315694	Peroxidase	0.992 Y	1	2	0	0	0	0	-
	SGN-U314196	Peroxidase	0.999 Y	9	22	1	1	1	1	-
	SGN-U327467	Peroxidase	0.000 N	2	2	0	0	0	0	-
	SGN-U314124	Peroxidase	0.994 Y	3	4	0	0	0	0	-
	SGN-U346809	Peroxidase	0.599 Y	1	1	0	0	0	0	-
Protease inhibiting proteins	SGN-U315605	Endoglucanase inhibitor protein	0.998 Y	0	0	2	2	3	3	-
	SGN-U317739	Protease inhibitor	0.006 N	8	16	7	19	8	30	-
	SGN-U312820	Protease inhibitor	0.966 Y	0	0	5	14	5	22	-
	SGN-U320265	Protease inhibitor	0.829 Y	3	4	4	8	6	9	-
	SGN-U313384	Protease inhibitor	0.998 Y	2	7	4	36	6	13	-
	SGN-U316036	Protease inhibitor	0.001 N	1	1	3	3	5	8	-
	SGN-U312826	Protease inhibitor	0.996 Y	2	2	4	4	5	10	-
	SGN-U312623	Protease inhibitor	0.000 N	2	2	0	0	5	6	-
	SGN-U315288	Protease inhibitor	0.916 Y	2	3	4	4	1	1	-
	SGN-U315879	Protease inhibitor	0.986 Y	4	7	4	7	4	7	-
	SGN-U322543	Protease inhibitor	0.000 N	5	12	7	19	1	3	-
	SGN-U320540	Protease inhibitor	0.003 N	0	0	1	1	2	2	-
	SGN-U312825	Protease inhibitor	0.996 Y	1	1	0	0	1	1	-
	SGN-U318506	Protease inhibitor	0.000 N	0	0	0	0	1	1	-
	SGN-U312828	Protease inhibitor	0.963 Y	0	0	0	0	1	2	-

Gene Id: identification number provided by the tomato protein database (ftp.sgn.cornell.edu/proteins/)
Putative function: was assigned based on sequence similarity when blasted using NCBI non redundant database
Signal P prediction value using algorithm SignalP3.0 server (http://www.cbs.dtu.dk/services/SignalP/)
Spectra: Total number of spectra matched to proteins in all replicates in an infected tomato fruit
Peptide: Total number of non-redundant peptides above a threshold score of 54 matched to the tomato protein in that infected tomato fruit
CAZy families: Predicted with CAZYmes Analysis Toolkit (CAT) from the BESC KnowledgeBase website (http://bobcat.ornl.gov/besc/index.jsp)

Table 2

Identified tomato cell wall-related proteins

Sub categorie	Gene Id	Putative Function	SignalP	MG		RR		*rin*		CAZy Families
				Peptide	Spectra	Peptide	Spectra	Peptide	Spectra	
	SGN-U320073	alpha-galactosidase	1.000 Y	-	-	1	1	-	-	GH27
	SGN-U319599	alpha-galactosidase	0.003 N	-	-	3	5	3	4	-
	SGN-U321036	alpha-glucosidase	0.000 N	-	-	2	3	3	4	GH31
	SGN-U319244	alpha-xylosidase	0.000 N	-	-	3	8	6	14	GH31
	SGN-U320295	alpha-xylosidase	0.001 N	1	1	-	-	7	10	GH31
	SGN-U320011	alpha-xylosidase	0.000 N	-	-	7	10	4	8	-
	SGN-U336993	alpha-xylosidase	0.000 N	1	1	4	5	3	4	-
	SGN-U312614	arabinogalactan protein	1.000 Y	2	8	4	24	7	24	-
	SGN-U322781	arabinogalactan protein	0.785 Y	3	7	4	8	10	11	-
	SGN-U320398	arabinogalactan protein	0.999 Y	3	7	3	3	4	5	-
	SGN-U316315	arabinogalactan protein	1.000 Y	-	-	-	-	1	1	GH18
	SGN-U319146	beta-D-glucan exohydrolase	0.000 N	-	-	-	-	1	1	GH3
	SGN-U345075	beta-D-glucan exohydrolase	0.015 N	-	-	-	-	1	1	-
Cell wall related proteins	SGN-U314662	beta-fructosidase	0.000 N	10	14	26	97	14	21	-
	SGN-U319861	beta-fructosidase	0.681 Y	-	-	-	-	5	11	GH32
	SGN-U319056	beta-galactosidase	0.020 N	-	-	4	4	-	-	-
	SGN-U315107	beta-galactosidase	0.013 N	-	-	2	3	-	-	-
	SGN-U322726	beta-galactosidase	0.362 N	-	-	2	2	-	-	-
	SGN-U328864	beta-galactosidase	0.000 N	-	-	1	1	1	1	-
	SGN-U316373	beta-Ig-H3 domain-containing protein unknown	0.811 Y	-	-	1	2	2	4	-
	SGN-U313742	endo-beta-mannanase	0.019 N	-	-	7	14	2	3	-
	SGN-U312953	expansin 1	0.998 Y	-	-	2	3	-	-	-
	SGN-U312510	expansin 10	1.000 Y	-	-	-	-	2	2	-
	SGN-U317347	expansin-like	1.000 Y	-	-	2	3	-	-	-
	SGN-U313100	extensin	0.000 N	-	-	6	8	2	4	-
	SGN-U322174	germin-like protein	0.999 Y	1	1	-	-	-	-	-
	SGN-U318102	germin-like protein	0.982 Y	1	1	4	6	1	2	-

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Sub categorie	Gene Id	Putative Function	SignalP	MG		RR		*rin*		CAZy Families
				Peptide	Spectra	Peptide	Spectra	Peptide	Spectra	
	SGN-U314144	glycosyl hydrolase	0.000 N	1	1	2	3	7	8	-
	SGN-U316037	glycosyl hydrolase	0.000 N	-	-	1	1	2	2	GH3
	SGN-U326255	hydroxyproline-rich glycoprotein	0.932 Y	-	-	1	1	-	-	-
	SGN-U318294	pectin acetylesterase	0.998 Y	1	2	8	26	11	28	-
	SGN-U312758	pectin esterase	0.002 N	-	-	21	114	14	68	CE8
	SGN-U313648	pectin esterase	0.001 N	-	-	5	6	9	17	-
	SGN-U318232	pectin esterase	0.252 N	-	-	4	10	4	10	-
	SGN-U313531	pectin esterase	0.992 Y	-	-	3	4	1	1	-
	SGN-U314637	pectin methylesterase	0.986 Y	-	-	3	5	-	-	-
	SGN-U312757	pectin methylesterase	0.966 Y	7	17	63	1039	55	861	CE8
	SGN-U313725	pectin methylesterase	0.009 N	2	2	14	66	11	87	-
	SGN-U313233	pectin methylesterase	0.156 N	-	-	-	-	1	1	CE8
	SGN-U330767	pectinacetylesterase	0.846 Y	-	-	1	1	-	-	CE13
	SGN-U320881	pectinacetylesterase	0.964 Y	-	-	1	1	1	1	CE13
	SGN-U324003	PG 2A precursor	0.999 Y	2	3	1	2	3	4	GH28
	SGN-U321477	polygalacturonase	0.000 N	1	1	-	-	-	-	-
	SGN-U312703	polygalacturonase	0.992 Y	8	10	63	435	23	59	GH28

Gene Id: identification number provided by the tomato protein database (ftp.sgn.cornell.edu/proteins/)
Putative function: was assigned based on sequence similarity when blasted using NCBI non redundant database
Signal P: prediction value using algorithm SignalP3.0 server (http://www.cbs.dtu.dk/services/SignalP/)
Spectra: Total number of spectra matched to proteins in all replicates in an infected tomato fruit
Peptide: Total number of non-redundant peptides above a threshold score of 54 matched to the tomato protein in that infected tomato fruit
CAZy families: Predicted with CAZYmes Analysis Toolkit (CAT) from the BESC KnowledgeBase website (http://bobcat.ornl.gov/besc/index.jsp)

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Table 3

Indentified *Botrytis cinerea* proteins

Sequence Id	Putative Function	Singal P		MG		RR		rin		Predicted CAZy Class
				Pep	Spec	Pep	Spec	Pep	Spec	
BC1G_07319.1	1,3-beta glucanase[CWDE]	0.999	Y	-	-	3	3	-	-	-
BC1G_10455.1	1,3-beta-glucanosyltransferase Gel1[CWDE]	0.998	Y	-	-	4	19	3	4	GH72
BC1G_02623.1	alpha-amylase	0.994	Y	-	-	2	2	4	5	GH13
BC1G_08975.1	alpha-fucosidase	0.962	Y	3	3	2	2	4	4	-
BC1G_12859.1	alpha-glucosidase precursor[CWDE]	1	Y	-	-	2	2	2	2	-
BC1G_08372.1	alpha-L-arabinofuranosidase A[CWDE]	0.995	Y	3	3	-	-	-	-	-
BC1G_04994.1	alpha-L-arabinofuranosidase[CWDE]	0.999	Y	-	-	-	-	2	2	GH54|CBM42
BofuT4_P078550.1	alpha-L-rhamnosidase B[CWDE]	0.072	N	-	-	-	-	1	1	GH78
BC1G_03983.1	beta-1,3 exoglucanase[CWDE]	0.27	N	-	-	2	2	-	-	-
BC1G_14030.1	beta-1-3-glucanosyltransferase	1	Y	3	4	-	-	3	4	GH72|CBM43
BC1G_03567.1	beta-galactosidase[CWDE]	0.998	Y	9	17	15	20	22	33	GH35
BC1G_10221.1	beta-glucosidase 1 precursor[CWDE]	0.816	Y	2	2	-	-	-	-	GH3
BC1G_02364.1	beta-glucosidase, putative[CWDE]	0.991	Y	5	7	-	-	-	-	GH3
BC1G_05538.1	beta-xylosidase[CWDE]	0.978	Y	1	1	-	-	-	-	GH3
BC1G_09079.1	cell wall beta-1,3-endoglucanase[CWDE]	0.999	Y	3	3	4	6	8	11	-
BC1G_14702.1	cellobiohydrolase 1 catalytic domain[CWDE]	1	Y	3	4	-	-	1	1	-
BC1G_06035.1	cellobiohydrolase[CWDE]	0.999	Y	12	21	-	-	-	-	GH7
BC1G_03188.1	cellobiose dehydrogenase[CWDE]	0	N	-	-	-	-	7	8	CBM1
BC1G_10880.1	cellobiose dehydrogenase[CWDE]	0.992	Y	17	57	-	-	12	19	GH7
BC1G_14012.1	choline dehydrogenase, putative	0.998	Y	-	-	2	2	-	-	CBM1
BC1G_08529.1	cytochrome c	0	N	-	-	-	-	2	2	-
BC1G_00576.1	endo-1,4 beta-D-xylanase[CWDE]	1	Y	2	2	2	3	-	-	GH10
BofuT4_P061530.1	endo-1,4-beta-mannosidase[CWDE]	0.998	Y	1	1	-	-	-	-	-
BofuT4_P124780.1	endo-glucanase, putative[CWDE]	0.999	Y	1	3	-	-	-	-	GH12
BC1G_11143.1	endo-polygalacturonase precursor[CWDE]	1	Y	15	57	13	87	25	161	GH28

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Sequence Id	Putative Function	Singal P		MG		RR		rin		Predicted CAZy Class
				Pep	Spec	Pep	Spec	Pep	Spec	
BofuT4_P024480.1	esterase	0.025	N	-	-	2	2	1	1	-
BC1G_07822.1	extracellular endo-glucanase[CWDE]	0.999	Y	3	3	-	-	-	-	GH5
BC1G_07496.1	extracellular exo-polygalacturonase, putative[CWDE]	0	N	2	3	-	-	-	-	GH28
BC1G_00448.1	extracellular serine-rich protein	0.983	Y	3	3	2	2	-	-	-
BofuT4_P151530.1	formate dehydrogenase	0.659	Y	-	-	1	1	3	4	-
BC1G_04836.1	fructose-bisphosphate aldolase	0	N	4	4	-	-	-	-	-
BofuT4_P119480.1	GDSL lipase/acylhydrolase family protein	0.988	Y	2	4	-	-	-	-	-
BC1G_04151.1	glucan 1,4-alpha-glucosidase	0.912	Y	5	7	11	15	20	84	CBM20|GH15
BC1G_10788.1	glucose oxidase	0.997	Y	1	1	-	-	-	-	-
BC1G_11898.1	glucosidase[CWDE]	0.999	Y	3	5	5	11	4	5	GH17
BofuT4_P033100.1	glutaminase GtaA	0.992	Y	-	-	3	3	-	-	-
BC1G_01204.1	glyoxal oxidase[CWDE]	0.983	Y	-	-	-	-	4	4	CBM18
BC1G_01393.1	hypothetical protein	1	Y	-	-	2	2	-	-	-
BC1G_02060.1	hypothetical protein	0.999	Y	1	2	2	3	-	-	-
BC1G_02930.1	hypothetical protein	0	N	-	-	7	11	-	-	-
BC1G_03976.1	hypothetical protein	0.959	Y	-	-	-	-	7	7	-
BC1G_05298.1	hypothetical protein	0	N	-	-	2	2	-	-	-
BC1G_05503.1	hypothetical protein	0	N	2	6	-	-	2	2	-
BC1G_05523.1	hypothetical protein	0	N	-	-	-	-	5	5	-
BC1G_05980.1	hypothetical protein	0.004	N	-	-	2	4	-	-	-
BC1G_07073.1	hypothetical protein	1	Y	-	-	2	2	-	-	-
BC1G_07275.1	hypothetical protein	1	Y	-	-	-	-	2	2	-
BC1G_07903.1	hypothetical protein	0.001	N	-	-	3	3	2	2	-
BC1G_08393.1	hypothetical protein	0.999	Y	3	5	-	-	2	4	-
BC1G_08615.1	hypothetical protein	0.962	Y	-	-	-	-	2	2	-
BC1G_08719.1	hypothetical protein	0.019	N	1	1	-	-	1	2	-
BC1G_10630.1	hypothetical protein	1	Y	3	4	-	-	-	-	-
BC1G_12374.1	hypothetical protein	0.999	Y	5	40	9	287	6	42	-
BC1G_15041.1	hypothetical protein	0.988	Y	4	5	-	-	4	5	-

Sequence Id	Putative Function	Singal P		MG		RR		rin		Predicted CAZy Class
				Pep	Spec	Pep	Spec	Pep	Spec	
BC1G_16040.1	hypothetical protein	0	N	-	-	-	-	1	1	-
BofuT4_P129550.1	hypothetical protein	0.998	Y	-	-	2	2	-	-	-
BC1G_12487.1	hypothetical protein	0	N	-	-	3	5	3	3	-
BC1G_14129.1	hypothetical protein	0.997	Y	2	4	2	2	2	4	-
BC1G_08553.1	laccase 2[CWDE]	0.999	Y	4	5	2	2	9	16	-
BC1G_10329.1	laccase[CWDE]	0.989	Y	-	-	4	6	-	-	-
BofuT4_P040250.1	L-PSP endoribonuclease family protein, putative	0	N	-	-	2	2	-	-	-
BC1G_00455.1	mannosyl-oligosaccharide alpha-1,2-mannosidase precursor[CWDE]	0.998	Y	4	5	-	-	2	2	GH47
BofuT4_P108020.1	nucleoside diphosphate kinase	0	N	1	6	1	3	2	3	-
BC1G_12017.1	pectin lyase A precursor[CWDE]	0.998	Y	-	-	3	8	3	3	PL1
BofuT4_P131540.1	pectin methyl esterase 1[CWDE]	1	Y	5	9	4	6	6	12	CE8
BC1G_00617.1	pectin methyl esterase[CWDE]	1	Y	2	4	-	-	2	5	CE8
BC1G_11144.1	pectin methylesterase, putative[CWDE]	1	Y	2	4	-	-	-	-	CE8
BC1G_13367.1	polygalacturonase 2 [CWDE]	0.999	Y	-	-	-	-	2	3	GH28
BC1G_00896.1	predicted protein	1	Y	5	39	5	44	6	87	-
BC1G_09084.1	predicted protein	1	Y	-	-	1	1	-	-	-
BC1G_14009.1	rhamnogalacturonan acetylesterase[CWDE]	0.983	Y	-	-	3	10	-	-	-
BofuT4_P124300.1	rhamnogalacturonan hydrolase [CWDE]	1	Y	1	1	-	-	1	1	GH28
BC1G_06836.1	serine protease	0.999	Y	-	-	3	4	-	-	-
BC1G_02163.1	SnodProt1[CWDE]	0.996	Y	13	65	27	253	19	154	-
BC1G_14403.1	thioredoxin	0	N	2	2	3	3	2	3	-
BofuT4_P021640.1	transaldolase 1	0	N	2	2	2	2	1	5	-
BC1G_00979.1	tripeptidyl-peptidase 1 precursor	0.015	N	1	1	-	-	1	2	-
BC1G_02944.1	tripeptidyl-peptidase 1 precursor	0.999	Y	-	-	-	-	2	2	-
BC1G_10797.1	xylosidase : arabinofuranosidase[CWDE]	0.997	Y	2	2	-	-	2	2	-

Sequence id: Identification number provided by the Broad Institute BO5.10 database
Putative function: was assigned based on sequence similarity whne blasted using NCBI non redundant database
Signal P: prediction value using algoritm SignalP3.0 server (http://www.cbs.dtu.dk/services/SignalP/); Y, signal peptide predicted. N,no signal peptide predicted.
Peptides: Total number of non-redundant peptides above a threshold score of 54 matched to the fungal protein in that infected tomato fruit
Spectra: Total number of spectra matched to proteins in all replicates in that infected tomato fruit

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CAZy families: Predicted with CAZYmes Analysis Toolkit (CAT) from the BESC KnowledgeBase website (http://bobcat.ornl.gov/besc/index.jsp)

Table 4

Common *Botrytis cinerea* proteins in secretome studies

Sequence Id	Putative Function	MG	RR	*rin*	O-glycosilation	N-glycosilation	Secretomes Studies
		Spectra	Spectra	Spectra			
BC1G_12374.1	hypothetical protein	40	287	42	none	Yes	This study, Sha et al 2009a, Sha et al 2009b, Espino et al., 2010, Fernández-Acero et al., 2010
BC1G_02163.1	SnodProt1	65	253	154	none	none	This study, Sha et al 2009a, Sha et al 2009b, Espino et al., 2010
BC1G_ 11143.1	endo-polygalacturonase precursor	57	87	161	none	none	This study, Sha et al 2009a
BC1G_00896.1	predicted protein	39	44	87	Yes	none	This study, Sha et al 2009a, Sha et al 2009b, Fernández-Acero et al., 2010
BC1G_03567.1	beta-galactosidase	17	20	33	none	Yes	This study, Sha et al 2009b, Espino et al., 2010
BC1G_04151.1	glucoamylase precursor	7	15	84	Yes	Yes	This study, Sha et al 2009a, Fernández-Acero et al., 2010
BC1G_11898.1	glucosidase	5	11	5	none	none	This study, Sha et al 2009a, Espino et al., 2010, Fernández-Acero et al., 2010
BC1G_09079.1	GPI-anchored cell wall beta-1,3-endoglucanase	3	6	11	Yes	Yes	This study, Sha et al 2009a, Sha et al 2009b, Espino et al., 2010
BofuT4_P131540.1	pectin methyl esterase	9	6	12	none	Yes	This study, Espino et al., 2010, Fernández-Acero et al., 2010
BC1G_ 14403.1	thioredoxin	2	3	3	none	Yes	This study, Sha et al 2009a, Sha et al 2009b
BofuT4_P108020.1	nucleoside diphosphate kinase	6	3	3	none	none	This study
BC1G_08553.1	laccase 2	5	2	16	none	Yes	This study, Espino et al., 2010
BC1G_08975.1	alpha-fucosidase	3	2	4	Yes	Yes	This study, Sha et al 2009b
BC1G_14129.1	hypothetical protein	4	2	4	none	Yes	This study
BofuT4_P021640.1	transaldolase 1	2	2	5	none	Yes	This study

Sequence id: Identification number provided by the Broad Institute BO5.10 database
Putative function: was assigned based on sequence similarity when blasted using NCBI non redundant database
Spectra: Total number of spectra matched to proteins in all replicates in that infected tomato fruit
O-glycosylation: Predicted with NetOGlyc 3.1 Server (http://www.cbs.dtu.dk/services/NetOGlyc/)
N-glycosilation: Predicted with NetNGlyc 1.0 Server (http://www.cbs.dtu.dk/services/NetNGlyc/)