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Proteomic Investigation of *Rhizoctonia solani* AG 4 Identifies Secretome and Mycelial Proteins with Roles in Plant Cell Wall Degradation and Virulence

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ABSTRACT: *Rhizoctonia solani* AG 4 is a soilborne necrotrophic fungal plant pathogen that causes economically important diseases on agronomic crops worldwide. Here we used a proteomics approach to characterize both intracellular proteins and the secretome of *R. solani* AG 4 isolate Rs23A under several growth conditions; the secretome being highly important in pathogenesis. From over 500 total secretome and soluble intracellular protein spots from 2-D gels, 457 protein spots were analyzed and 318 proteins positively matched with fungal proteins of known function by comparison with available *R. solani* genome databases specific for anastomosis groups 1-IA, 1-IB, and 3. These proteins were categorized to possible cellular locations and functional groups; and for some proteins their putative roles in plant cell wall degradation and virulence. The majority of the secreted proteins were grouped to extracellular regions and contain hydrolase activity.

KEYWORDS: *Rhizoctonia solani* AG 4, 2D-proteomics, secretome, virulence

■ INTRODUCTION

Rhizoctonia solani sensu lato (Teleomorph: *Thanatephorus cucumeris* [Frank] Donk) is a genetically heterogenous soilborne fungus that can cause economically important diseases worldwide.¹ Pathogenic isolates of this fungus are known to attack at least 188 species of higher plants, including cereal crops, vegetables, ornamentals, forest trees, and turfgrasses.¹ These isolates of *R. solani* are responsible for pre- and post-emergence damping-off of seedlings, crown necrosis, and root rots; accounting for up to 20% in yield and quality losses in the United States.² *R. solani* is considered a species complex with subgroupings traditionally based on

hyphal anastomosis reactions.³ There are at least 13 anastomosis groups (AGs) of *R. solani* described in the literature with isolates from each AG maintaining distinct host range specializations, epidemiology, and sensitivity to pesticides.³ Isolates from AG 4 cause important diseases on plants worldwide while isolates from other AGs are typically less destructive to plants and more limited in geographic distribution.³ Isolates from AG 4 are now classified as a distinct *Rhizoctonia* species, *Thanatephorus praticola*.¹

Pathogenic isolates from the *R. solani* species complex, including those from AG 4, are necrotrophic, acquiring nutrients for colonization of plant tissues from dead or dying plant cells. It is likely that there is also a very brief biotrophic phase during pathogenesis where host recognition occurs.⁴ Events occurring at the macro- and micro-scopic level during plant infection by isolates from AG 4 include adhesion to plant surfaces, production of cushion infection structures, killing of plant cells before or immediately after penetration of plant tissue, and subsequent colonization of degraded or moribund tissue.¹ However, processes occurring at the molecular level that lead to these events as well as the host plant response, soil adaptability, and pesticide sensitivity are poorly characterized due to a dearth of studies at the molecular level with isolates from AG 4. Gene expression profiling was used to identify several cDNAs with potential roles in fungal pathogenicity, virulence, and signal transduction from an *R. solani* AG 4 isolate.⁵ The genomes of *R. solani* AG 3 isolate Rhs IAP, AG1-IA, AG1-1B isolate 7/3/14, and AG 8 have also recently been sequenced.⁶⁻⁹ However, due to genomic variation among AGs of *R. solani*, findings from genomic studies with isolates from other AGs may not be directly applicable to our understanding of the molecular basis of plant disease caused by isolates from *R. solani* AG 4 and their ecology.

Here we developed proteomic profiles of extracellular and mycelial proteins of *R. solani* AG 4 (HG-I) isolate Rs23A to further characterize isolates from AG 4 at the molecular level. We analyzed a total of 457 protein spots from combined mycelial and secretome protein profiles of Rs23A in 2-D gels, of which 225 protein spots representing 318 proteins were matched with fungal proteins of known function. These proteins were categorized to possible cellular locations and functional groups; and for some proteins, the putative roles in pathogenesis and ecology identified.

▪ MATERIALS AND METHODS

***Rhizoctonia solani* Isolate.** *Rhizoctonia solani* AG 4 (HG-I) isolate Rs23A¹⁰ was obtained from the USDA-ARS, Floral and Nursery Plants Research Unit culture collection and maintained by periodic culturing on Potato Dextrose Agar (PDA; Sigma Aldrich Co., St. Louis, Missouri) or by storage of inoculated wheat or barley seeds in 20% glycerol at -80 °C.⁵

Extraction of Extracellular Proteins from Isolate Rs23A Grown in the Presence of a Soybean Seedling. Isolate Rs23A was grown in 200 mL of medium #1) 1X Vogel's minimal salts medium containing nitrogen (Medium N)¹¹ plus 1 g asparagine L⁻¹, 14 mM glucose, and a soybean seedling; medium #2) the same medium as #1 with the exception that 112 mM glucose was used; and medium #3) the same medium as #2 with the exception that no soybean seedling was added. Isolate Rs23A was first grown on PDA plus 4% lactic acid prior to inoculation of 200 mL medium with six fresh 5-mm-diameter plugs of mycelia. Axenically germinated soybean seedlings (3 seedlings per bag) were added to the appropriate flasks in dialysis bags

91 (MWCO = 100 kDa; Cat. No. 131417; Spectrum Laboratories Inc., Rancho Dominguez,
92 California) 2 days after incubation of Rs23A in the above media in the dark at 15 rpm and 22 °C.
93 Cultures were subsequently incubated 7 days without shaking. Cultures were filtered through 2
94 MM Whatman filter paper and the filtrate lyophilized and suspended in 5 mL PBS containing 3
95 mM dithiothreitol (DTT), 500 μ L PMSF, and 10% glycerol. Samples were then dialyzed
96 overnight (100 kDa MWCO) in 10 mM TrisCl, pH 7.0, plus 3 mM EDTA, and concentrated in
97 Amicon Ultra-15 centrifugal filter units containing an Ultracel-3 membrane (Millipore
98 Corporation, Billerica, Massachusetts). Concentrated protein samples were precipitated in 4 X
99 ice-cold precipitation solution (10% TCA and 0.07% DTT in acetone) at -20 °C for 1 h and
100 precipitated protein collected by centrifugation at $14,000 \times g$ for 20 min at 4 °C. Pellets were
101 rinsed twice by vortexing in acetone containing 0.07% DTT and incubated at -20 °C in this
102 solution for 30 min followed by centrifugation as above. Vacuum-dried pellets were solubilized
103 by sonicating in 0.6 mL IEF solution for 10 min. Protein solutions were stored at -80 °C until
104 used in 2-D gel electrophoresis.

105

106 **Extraction of Extracellular Proteins from Isolate Rs23A Grown on Citrus Pectin.** Isolate
107 Rs23A was grown in 100 mL medium #4, which is medium #1 (above) with citrus pectin (0.5 g
108 L^{-1} ; Sigma Chemical Co.; Cat. No. P9135) 5 to 7 days at 22 °C in the dark. As a control, Rs23A
109 was also grown in medium #1 with the exception that 112 mM glucose was used. Cultures were
110 centrifuged at $10,000 \times g$ for 20 min at 4 °C and the supernatant sequentially filtered through
111 autoclaved Whatman No. 1 and 0.45 μ filters, collected on a siliconized glass vessel, stored at -
112 80 °C for 24 h, thawed, and passed through two layers of autoclaved Whatman No. 1 filters.

This filtered supernatant was extracted with an equal volume of Tris-HCl (pH 7.8-8.0) buffered phenol by vortexing for 30 min in the cold room (4 °C). The phenol phase was separated by centrifugation at $5000 \times g$ for 15 min at 4 °C. To precipitate protein, 5 volumes ice-cold ammonium acetate-saturated methanol was added to the phenol phase, mixed by vortexing, and incubated overnight at -20 °C. The precipitate was collected by centrifugation at $5,000 \times g$ for 30 min at 4 °C, and washed twice with cold ammonium acetate-saturated methanol and twice with ice cold 80% (v/v) acetone. The pellet was resuspended by sonication and vortexing in 0.6 mL IEF buffer as above. Protein solutions were stored at -80 °C until used in 2-D gel electrophoresis.

Extraction of Mycelial Proteins. Isolate Rs23A was grown in Potato Dextrose Broth (medium #5) in 500 mL flasks at room temperature in the dark for 7 days. Total mycelial proteins were extracted using the phosphate solubilization procedure followed by TCA precipitation.¹⁰

Two-Dimensional Gel Resolution of Extracted Proteins. Extracted proteins were resolved in 2-D gels as reported previously.¹⁰ In brief, about 500 µg of extracted protein was loaded onto an IPG strip and run at 20 °C at 30 V for 14 h, 500 V for 1 h, 1000 V for 1 h, and finally at 8000 V until 25 kVh was achieved for pH 4-7 strips; and 30 V for 13 h, 500 V for 1 h, 1000 V for 1 h, and finally at 8000 V until 30 kVh was achieved for pH 6-11 strips. Proteins were resolved in the second dimension on 12.5% SDS-PAGE gels (18 × 16 cm) in Tris-glycine SDS buffer on a Hoefer SE 600 Ruby electrophoresis unit (GE Healthcare, Pittsburg, PA) following the manufacturer's recommendations. Gels were fixed in 45% ethanol and 10% acetic acid, equilibrated in 34% methanol, 17% ammonium sulfate, and 3% phosphoric acid, stained in

Coomassie Blue G-250, and scanned with laser densitometry in a PDSI scanner (GE Healthcare).¹⁰ Extracellular proteins were resolved with IEF at pH 4-7 while intracellular proteins were resolved with IEF at pH 4-7 and pH 6-11.¹⁰ A total of two pH 4-7 and two pH 6-11 2-D gels were run for mycelial proteins and two pH 4-7 2-D gels each were run for the glucose alone, soybean-induced, and pectin-induced secretomes. Extractions were repeated and 2-D gels run on the second extract preparations as just described. One representative 2-D gel for each extraction was used to estimate the total number of protein spots.

Protein Identification. Tryptic peptide fragments from gel spots from extracellular protein extracts were analyzed with either an AB SCIEX TOF/TOF™ 5800 System (AB SCIEX Pte., Ltd., Framingham, Massachusetts) operated in positive ion reflector mode, or via an LTQ Orbitrap XL mass spectrometer (ThermoFisher Scientific Inc., San Jose, California) as described.¹² For the identification of intracellular proteins, peptides were analyzed with an LCQ DECA XP mass spectrometer (ThermoFisher Scientific Inc.) as described.¹³ All MS/MS samples were analyzed using Mascot software (Matrix Science, London, United Kingdom; version 2.3.02). A species specific FASTA formatted database was constructed for Mascot searching by downloading the protein sequence subset from the National Center for Biotechnology Information non-redundant (NCBI nr) protein database using the taxonomy filter *R. solani*, for a total of 35,849 sequences (339 from *R. solani*; 12,726 from *R. solani* AG-3 Rhs1AP; 12,268 from *R. solani* AG-1 IB; and 10,516 from *R. solani* AG-1 IA) as of 04/14/14. The FASTA formatted sequences of porcine trypsin and common human keratin contaminants were appended to the database for a total of 35,861 sequences. Additionally, FASTA formatted databases were downloaded from NCBI nr for *Glycine max* and the metallobacterium

Cupriavidus metallidurans to search for, and exclude, contaminating proteins from these two species. We detected soybean proteins and *C. metallidurans* proteins (possible contaminant from citrus pectin) in certain preparations of the *R. solani* secretome. Scaffold (version Scaffold_4.3.0; Proteome Software Inc., Portland, Oregon) was used to validate TOF/TOF based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 80.0% probability as specified by the Peptide Prophet algorithm.¹⁴ Protein probabilities were assigned by the Protein Prophet algorithm.¹⁵ Protein identifications were accepted if they could be established at greater than 95.0% probability and contained at least two identified peptides, except for the glucose-induced (medium #1, FIG. 1, panel A) secreted proteins for which, a single peptide match was also accepted. The rationale was that 1) no protein source was included in medium #1, 2) a high resolution TOF/TOF was used and selected for high ion score peptide detection, 3) peptides were screened through two levels of security (MASCOT raw ion score and Peptide Prophet algorithm in Scaffold), and 4) the databases searched were closely related (i.e. *R. solani* AG1-IA, AG1-IB, and AG 3), but still different from *R. solani* AG 4 at the species level.¹ The accuracy of using single peptide matches in protein identification has been discussed.¹⁶

The *FunSecKB* database was used¹⁷ for comparing the homology of identified secretory proteins. The *FunSecKB* database utilizes SignalIP, WolfPsort, and Phobius for prediction of signal peptides or subcellular location, TMHMM for identifying membrane proteins, and PS-Scan for identification of endoplasmic reticulum target proteins.¹⁷ For identification of fungal plant-cell-wall-degrading enzymes, the secretome was searched against the Fungal Plant-Cell-Wall-Degrading Enzymes database (FPDB).¹⁸ Homologues of fungi specific virulence factors

were identified with the Database of Fungal Virulence Factors (DFVF).¹⁹ GO ontologies were determined using Blast2GO.²⁰

▪ RESULTS

General Characteristics of the Rs23A Secretome Dataset. We initially selected 89 protein spots reproducibly detected in the 2-D gels of the secretome when isolate Rs23A was grown on medium #1 (FIG. 1 panel A). Five protein spots were corrupted during MS/MS analysis and 23 protein spots were contaminated with keratin and trypsin reducing our analysis of the Rs23A secretome when this isolate was grown on medium #1 to 61 protein spots. Spots 4, 5, 6, 12, 13, 18, 23, 38, 40, 44, 51, 86, and 87 each had two protein matches, and spots 14 and 22 each had three protein matches in the *Rhizoctonia* genome databases, resulting in the identification of 80 proteins altogether. Of these 61 protein spots, 43 (71%) were identified using the three existing *R. solani* genome data bases. This high percentage of functional annotation of secretome proteins was significant in comparison to non-annotated and some annotated fungal genomes.²¹ Only 18 (29%) of the proteins were designated as hypothetical proteins (non-annotated) (spots 7, 27, 28, 29, 30, 38, 39, 40, 48, 51, 75, 80, 81, 82, 83, 84). All proteins except spot 65 (Hemopexin domain-containing protein), spot 75 (hypothetical protein), and spot 83 (hypothetical protein) had corresponding matches in the *FunSecKB* database, indicating that there was minimal, if any, contamination of the Rs23A secretome with intracellular proteins. Even the Hemopexin domain-containing proteins in animal and bacterial cells have been shown to be secreted proteins. However, 'moonlighting' (multifunctional) roles of some of the proteins

could not be excluded. In several other papers regarding fungal secretome studies, intracellular protein contamination was found to be an unavoidable part of the protein analysis.²¹

We selected 108 and 86 protein spots that were reproducibly detected in the 2-D gels from isolate Rs23A grown in the presence of soybean (medium #2, FIG. 2 panels A, B) and pectin (medium #4, FIG. 1 panel B), respectively. However, a total of 31 and 7 *R. solani*-specific protein spots were used after exclusion of soybean proteins and *C. metallidurans* proteins, respectively (Appendix TABLE S1). We detected a total of 35 proteins from the 31 spots (FIG. 2 panels A, B, C) from the soybean induced secretome experiment, as some of the protein spots (4, 20, 30, 31) were identified as comigrating mixtures of two different *R. solani* proteins. Of these 35 proteins, only 4 were identified as hypothetical proteins, and 33 proteins had homologues in secretome database (FunSecKB), 15 proteins had homologues in virulence database (DFVF), and 7 had homologues in the FPDB.¹⁸ Of the 7 protein spots detected in the secretome of Rs23A grown in the presence of pectin, 7 had homologues in the secretome database, 2 had homologues in the virulence database, and 6 had homologues in the FPDB. A very low percentage of proteins in the Rs23A secretome when this isolate was grown in the presence of soybean or pectin were not identified (hypothetical proteins) as was the case with proteins detected in the secretome of Rs23A grown on glucose only. All proteins from soybean-induced and pectin-induced preparations were detected in the secretome database (FunSecKB) suggesting again, that there was minimal contamination of the Rs23A secretome with intracellular protein.

From the combined 122 secreted proteins from the five cultural conditions, 96 proteins with homology in the GO database were organized into functional categories for (1) biological process, (2) molecular function and (3) cellular components (FIG. 3). The two major biological process functional groups were the primary and the organic substance metabolic processes,

respectively. The three major molecular functions of the Rs23A secretome were hydrolase activity, ion binding, and carbohydrate binding activities, respectively.

The growth conditions in these different media led to different secretome profiles as visualized after 2-D gel separation and staining (FIGs. 1 and 2) showing a clear response of the Rs23A secretome to the different media components. It should be noted that majority of the protein spots in the citrus pectin-induced *R. solani* secretome preparation were *Cupriavidus metallidurans* proteins potentially a contaminant in the commercial pectin source.

Rs23A Secretome Protein Identification. Proteins identified in secretome extracts from Rs23A grown in medium #1 are listed in Appendix TABLE S1. The majority of proteins had homologues in the FPDB, consistent with the necrotrophic lifestyle of this plant pathogen. Of these cell-wall-degrading enzymes, a large number belonged to glycoside hydrolase (GH).²² This was followed in number by hypothetical proteins and extracellular invertases. GHs are the most diverse group of enzymes used by microbes in the degradation of biomass. In a recent comparative analysis of plant-cell-wall-degrading enzymes of fungal genomes, of the total 127 GH families, 91 were detected in fungi, with the most prevalent families being GH5, GH13, GH31, and GH61.²² Rs23A secretome proteins identified in our study belonged to GH family 6 (cellobiohydrolase and endoglucanase) (spots 31, 32), GH family 13 (α -glucosidase, α -amylase, and oligo-1,6-glucosidase) (spots 10, 12, 13, 16, 18, 20, 21, 22, 23), GH family 15 (glucoamylase) (spot 6, soybean-induced spot 8, pectin-induced spots 3, 4, 5, 6), GH family 31 (α -glucosidase, α -xylosidase, and invertase) (spots 5, 6, 10, 11, 12, 13, 14, 15, 17, 19, 24, 25, 86, 87, 88; soybean-induced spots 4, 5, 6, 11), and GH family 92 (mannosidase) (spots 85, 86, 87). Other secretome proteins identified in our study were glucoamylase (spots 14, 22), α -amylase

(spots 8, 9), pathogenesis-related protein (thaumatin family) PR5K (spot 54), mannosidase (spots 85, 86, 87), carbohydrate esterase (spot 66), extracellular invertase (spots 18, 44, 45, 47, 51, 53), extracellular metalloproteinase MEP (spots 36, 40), GDSL-like lipase/acylhydrolase (spots 37, 38), lipase (spots 41, 49), trehalase (spots 1, 2, 4), peptidyl-lys metalloendopeptidase (spot 77), copper radical oxidase (spot 5), and SH3 domain-containing protein (spot 56).

Several secretome proteins (spots 1, 2, 4, 5, 6, 10, 11, 12, 13, 14, 15, 17, 18, 19, 24, 25, 36, 40, 41, 49, 66, 86, 87, 88) had corresponding fungal virulence factor homologues in the DFVF database.¹⁹ The majority of these proteins were α -glucosidases (GH family 31) although proteins belonging to GH families 6, 13, 15, and 92 were identified. Several GH families have been shown to be fungal virulence factors in plants.²² Other proteins identified as virulence factors were glucoamylase (spot 14), carbohydrate esterase family 4 protein (spot 66), mannosyl-oligosaccharide α -1,2-mannosidase (spots 86, 87), extracellular metalloproteinase MEP (spots 36, 40), extracellular invertase (spot 18), lipase (spots 41, 49), trehalase (spots 1, 2, 3), and copper radical oxidase (spot 5). PR5K (spot 54) is a β -1,3-glucanase belonging to GH family 64. In plants, PR5K is involved in acquired systemic resistance, causing the inhibition of hyphal growth and reduction of spore germination, probably by interaction with pathogen receptors.²³ Carbohydrate esterase family 4 proteins (spot 66), also known as acetylxylen esterases, are components of hemicellulolytic and cellulolytic enzyme systems of microorganisms functioning in the utilization of plant biomass. They de-esterify partially acetylated hardwood 4-*O*-methyl-D-glucuronoxylan and xylans of annual plants.²⁴ Metalloproteases (spots 36, 40), which contain zinc as an essential metal ion for the catalytic activity, are the most diverse of the four main types of protease, and have been determined to play important roles in the pathogenicity of fungi, including vertebrate pathogens, entomopathogens, and phytopathogens. In the rice plant

pathogen *Magnaporthe grisea*, a metalloprotease was shown to function as a gene-for-gene-specific avirulence factor by directly binding the plant resistance gene product and triggering a signaling cascade leading to resistance.²⁵ Lipase (spots 41, 49) has been shown to be a major virulence factor in the plant pathogenic fungus *Fusarium graminearum*.²⁶

The trehalose pathway (spots 1, 2, 3) is essential for stress tolerance and virulence in fungi. In the plant pathogenic fungus *Magnaporthe grisea*, trehalose biosynthesis genes are able to regulate expression of nitrogen source utilization, and direct virulence genes.²⁷ Copper radical oxidases (spot 5) are known to influence virulence of *Ustilago maydis* in plants.²⁵ We also found some of hypothetical proteins that have homologues in the DFVF (spots 12, 27, 29, 40).

Proteins identified in secretome extracts from Rs23A grown in the presence of soybean (soy-induced; medium #2) and pectin (pectin-induced; medium #4) are listed in Appendix TABLE S1. All of the virulence-related proteins in the secretome when Rs23A was grown in the presence of soybean were detected in the secretome of this isolate grown on medium #1 (see above) except soy-induced spots #20 and #24. Glucose-methanol-choline (GMC) oxidoreductase (soy-induced spot 20) when produced by *Glomerella cingulata* was shown to play a role in plant pathogenicity through the reduction of antifungal pathogen-induced quinones and phenoxy radicals that function in the reinforcement of plant cells walls during pathogen attack.²⁸ β -1,3-glucan synthase (soy-induced spot 24) is a glucosyltransferase enzyme involved in the generation of β -glucan in fungi. β -1,3-glucan and chitin are the most prominent fungal cell wall polysaccharides. In RNAi strains of *C. graminicola*, downregulation of β -1,3- glucan synthesis in appressoria increased cell wall elasticity and bursting of these appressoria.²⁹

Several proteins in the secretome of Rs23A grown on citrus pectin were not detected in the secretome of this isolate grown on medium #1 (Appendix TABLE S1). These proteins were

laccase (pectin-induced spot 1), α -galactosidase (GH family 27) precursor (pectin-induced spot 7), α -glucosidase (pectin-induced spot 2), glucoamylase (GH family 15) and carbohydrate-binding module family 20 (pectin-induced spots 3, 4, 5, 6). The role of α -glucosidase and GH family 15 proteins were described above. Laccases play a role in the formation or degradation of lignin and are important virulence factors for plant pathogenic fungi, presumably due to their detoxification of defense metabolites produced by plant hosts.²⁸ α -galactosidase was found to be essential for appressorial growth and pathogenicity of *Ustilago maydis*.³⁰

General Characteristics of the Rs23A Intracellular Protein Dataset. Altogether 152 protein spots were picked for MS/MS analysis from 2-D gels with IEF performed at pH 4-7 and 36 spots from 2-D gels with IEF performed at pH 6-11 (FIG. 4 panels A and B). Of these 188 protein spots, 62 protein spots were contaminated with trypsin and keratin and were excluded from the final analysis. From the 126 protein spots remaining in the analysis, 6 proteins were *R. solani*-specific hypothetical proteins.

Multiple *Rhizoctonia*-specific proteins with high peptide matches were detected from some spots. For example, pH 4-7 gel spots 24, 26, 34, 44, 45, 48, 59, 60, 61, 69, 74, 81, 90, 91, 92, 94, 96, 137, 141, 142, 144, 145, 146, 151, 152, and pH 6-11 gel spots 1, 8, 9, 13, 15, 22, 27, 32, and 33 had 2 proteins detected from within each spot. Similarly, three proteins were detected from within each of the pH 4-7 gel spots 65, 70, 72, 73, 97, 147, 149, and pH 6-11 gel spots 25, 31, 34, 35, 36. Four proteins were detected from within each of pH 4-7 gel spot 93 and pH 6-11 gel spots 23, 28 and 30. Thus, altogether 196 *Rhizoctonia*-specific proteins were detected from the intracellular proteome. The intracellular proteins with homology in the GO database (level 3) were functionally grouped for (1) biological process, (2) molecular function and (3) cellular

components (FIG. 5). The major four categories of proteins of biological process belonged to organic substance-, cellular-, primary-, and single organism-metabolic processes. At the molecular function level, the four major functional categories were ion binding, organic cyclic compound binding, heterocyclic compound binding, and oxidoreductase activity. The three major groups at the cellular component levels were cell part, protein complex, and membrane-bounded organelle. The combined intracellular and secretome also showed very similar trends at the biological process, molecular function, and cellular component levels. Functional grouping of combined intracellular and secreted proteins are presented in Appendix FIG S1. Organization of these proteins at level 4 is presented in the supplementary information [Appendix FIG. S2 A (secreted), B (intracellular), C (combined secreted and intracellular)]. Proteins identified in the intracellular extract from Rs23A are listed in Appendix TABLE S2.

Plant-Cell-Wall-Degrading Enzymes and Pathogenicity or Virulence-Associated

Intracellular Proteins. Of the intracellular proteins identified from Rs23A (Appendix TABLE S2), at least 5 proteins had homologues in the FPDB database and 47 of these intracellular proteins had homologues in the fungal virulence factor database (DFVF). The heat shock 70 kDa protein (HSP70; IEF pH 4-7, spots 14, 15, 140) was among these pathogenesis-related proteins. HSP70 has been shown to be a molecular chaperone essential for tolerance to protein denaturing stress and protein biogenesis. HSP70 may play a role in pathogenesis of coffee by the fungus *Colletotrichum gloeosporioides* by facilitating pathogen infection.²⁸ The eukaryotic initiation factor-5A (eIF-5A; IEF pH 4-7, spot 104), following posttranslational activation, was shown to transport a subset of mRNAs out of the nucleus to ribosomes for translation. eIF-5A has been the only hypusine-containing eukaryotic initiation factor identified so far. CNI-1493,

341 an inhibitor of hypusination, prevented germination and virulence of the wheat scab fungus
342 *Fusarium graminearum* on maize.³² The hemicellulose-degrading enzyme GH31/ λ -glucosidase
343 (IEF pH 4-7, spots 48, 149) was shown to be a cell-wall-degrading enzyme.²² We detected a
344 GH31/ λ -glucosidase homologue intracellularly and in the *R. solani* Rs23A secretome. Thiamine
345 biosynthesis (IEF pH 4-7, spots 35, 36, 37) genes were one of the most common Alternate
346 Splicing-enriched Pfam-coding gene families associated with fungal multicellular complexity
347 and virulence.³³ The homodimer fructose-bisphosphate aldolase (IEF pH 4-7, spots 62, 63), a
348 Class II aldolase, has been shown to catalyze the cleavage of fructose 1,6 bisphosphate by using
349 a zinc ion as an electrophile. Class II aldolases, found predominantly in fungi, have been
350 considered useful targets for discovering antifungal and antiprokaryote agents.³⁴ Thiolases (IEF
351 pH 4-7, spots 94, 95; IEF pH 6-11, spots 27, 28, 36), also known as acetyl-coenzyme A
352 acetyltransferases (ACAT), function in β -oxidation of fatty acids and were important for the
353 utilization of storage lipids or exogenous fatty acids and for the generation of acetyl coenzyme A
354 (acetyl-CoA). Defects in β -oxidation were shown to influence virulence in the phytopathogenic
355 fungi *Leptosphaeria maculans*, *Ustilago maydis*, and appressorium-based invasion in
356 *Magnaporthe grisea*, *Colletotrichum* species, and *Alternaria alternate*.³⁵ Glutathione reductase
357 (GR) (IEF pH 4-7, spots 72, 97; IEF pH 6-11, spot 31) also known as glutathione-disulfide
358 reductase (GSR) catalyze the reduction of glutathione disulfide to the sulfhydryl form (GSH)
359 which was shown to be a critical molecule in resisting oxidative stress and maintaining the
360 reducing environment of the cell. Loss of glutathione reductase and thioredoxin reductase
361 (NADPH) (IEF pH 4-7, spot 60) by *M. oryzae* resulted in strains being severely attenuated in
362 their ability to grow in rice cells, their failing to produce spreading necrotic lesions on the rice
363 leaf surface, and their failing to neutralize plant generated reactive oxygen species (ROS).³⁶

364 Aryl-alcohol oxidase (IEF pH 4-7, spots 49, 152) was shown to be an extracellular flavoprotein
365 providing the H₂O₂ required by ligninolytic peroxidases for fungal degradation of lignin.³⁷
366 Translational endoplasmic reticulum (ER) ATPase (IEF pH 4-7, spot 16), functioned in the
367 unfolded protein response (UPR), an essential part of ER protein quality control that ensured
368 efficient maturation of secreted and membrane-bound proteins in eukaryotes. In the absence of a
369 functional UPR, *Alternaria brassicicola* lost its ability to secrete the arsenal of toxic
370 compounds that it used for virulence, became hypersensitive to plant antifungal defense
371 compounds, and was avirulent.³⁸ Peptidyl-prolyl cis-trans isomerase or cyclophilin 1 (IEF pH 6-
372 11, spots 7, 8, 9) had peptidyl prolyl cis-trans isomerase activity that performed protein folding
373 by catalyzing the isomerization of peptide bonds that preceded proline residues. Cyclophilins
374 were reported as important virulence factors in infection of rice by *M. grisea* and in later
375 infection stages or plant colonization by *B. cinerea*.²¹ The *N*-acetyl- γ -glutamyl-phosphate
376 reductase (acetylglutamate kinase) (IEF pH 6-11, spot 33) of the crucifer anthracnose fungus
377 *Colletotrichum higginsianum* was reported to be a pathogenicity factor.³⁹ The ubiquitin / 40S
378 ribosomal protein S27a (pH 6-11, spot 1) and the 26S proteasome (IEF pH 4-7, spot 138; IEF pH
379 6-11, spot 28) function in the Ubiquitin-Proteasome System which has been shown to play a role
380 in virulence of *F. graminearum*, *F. oxysporum*, and *M. oryza*, and in sexual reproduction of
381 several plant pathogenic fungi.⁴⁰ Although not found in the DFVF database, diene lactone
382 hydrolase (pH 4-7, spots 79, 80) has recently been shown to have virulence role in the cereal
383 plant pathogenic fungus *Fusarium pseudograminearum*.⁴¹

384

385 **Other Biologically Relevant Intracellular Proteins.** Although not related to fungal virulence,
386 several identified intracellular proteins were found that are associated with fungal ecology and

soil health, cellular metabolism, growth and development, or that are possible future therapeutic targets. Hemopexin (or haemopexin, HPX; IEF pH 4-7, spots 34, 81, 83, 90, 143, 144, 145, 146, 147, 148; IEF pH 6-11, spots 1, 15, 16), also known as β -1B-glycoprotein, belongs to the hemopexin family of proteins. Although similar to siderophores in function, hemopexin binds heme with the highest affinity of any known protein. Thus, in low iron environments, the presence of hemopexin domain-containing proteins may serve as a backup system for iron chelation. HSP60 (pH 4-7, spots 18, 19) plays a role in mitochondrial protein import and macromolecular assembly. Also, a proteinaceous soil compound glomalin was found to be homologous to HSP60 in the arbuscular mycorrhizal fungus *Glomus intraradices*. The presence of glomalin was highly correlated with soil aggregate water stability.⁴² Thus, the role of HSP60 in the soil ecology of *R. solani* warrants future investigations. Rho-GDP dissociation inhibitors (Rho-GDI) (pH 4-7, spot 30) are repressors of Rho-type monomeric GTPases that control fundamental cellular processes, such as cytoskeletal arrangement, vesicle trafficking, and polarized growth. Profilin (pH 4-7, spot 99) is important for spatially and temporally controlled growth of actin microfilaments, which is an essential process in cellular locomotion and cell shape changes, including growth of fungal tips.⁴³ Homocysteine S-methyltransferase (pH 6-11, spot 34), also known as methionine synthase, transfers a methyl group from a donor to L-homocysteine. Mammals use a cobalamin cofactor. However, in all fungi the methyl group is transferred directly from 5-methyltetrahydrofolate-glutamate³, forming methionine and tetrahydrofolate. The fungal enzyme is cobalamin independent and structurally different from the mammalian counterpart; thus being a potentially important therapeutic target.⁴⁴ Import receptor subunit tom40 eukaryotic porin Tom40 (pH 6-11, spot 28) is a channel-forming protein essential for import of protein precursors into mitochondria.

DISCUSSION

Plant infection by fungal necrotrophs, such as *R. solani* AG 4 isolate Rs23A, is a complex process where attachment of the infective propagule to the plant surface is followed by penetration, lesion formation and expansion, and maceration of plant tissues; providing the pathogen access to nutrients and water. During this process the pathogen is first confronted by the external hydrophobic surface of the plant comprised of cutin or suberin and then by the plant cell wall. Suberin and cutin are lipid structures formed from hydroxy or epoxy fatty acids joined by ester linkages while the plant cell wall is a complex, dynamic structure containing intertwined networks of polysaccharide, lignin, and protein.⁴⁵ Cellulose, hemicellulose, and pectin, the major polysaccharides in the plant cell wall, contain a diverse collection of monosaccharides and linkages requiring an equally diverse collection of cell-wall-degrading enzymes for their degradation.⁴⁵ Analysis of the secretome of *R. solani* Rs23A revealed lipases, potentially having activity on the cutin or suberin outercoating of the plant. Additionally, a complex arsenal of secreted cell-wall-degrading enzymes was detected in the secretome; a large number of these cell-wall-degrading enzymes belonging to at least five glycoside hydrolase (GH) families. It is these diverse GHs that degrade the main chains of plant polysaccharides, potentially having the greatest impact on the degradation of cellulose, hemicelluloses, and lignocellulose.⁴⁶ Other enzymes detected in the Rs23A secretome which can potentially aide in degradation of the plant cell wall by this pathogen are acetylxytan esterases and metalloproteases, by targeting xytan, a component of hemicellulose, and protein in the plant cell wall. Copper radical oxidases, aryl-alcohol oxidase, and laccase, which function in lignin-degradation^{37, 22} were also detected.

Degradation of these plant structures during pathogenesis releases plant elicitors that lead to modification and reinforcement of the plant cell wall through the deposition of callose, accumulation of phenolics, and synthesis of lignin. Proteinaceous inhibitors of cell-wall-degrading enzymes and antimicrobial compounds such as phytoalexins are formed as well.⁴⁵ There are examples in the literature of fungal necrotrophs aiding infection through chicanery, such as by coercing the plant into altering the plant cell wall to provide more accessibility to cell-wall-degrading enzymes.⁴⁵ It is interesting to speculate that the Rs23A PR5K (spot 54) functions in infection by mimicking the plant PR5K counterpart. In plants, PR5K is involved in acquired systemic resistance, causing the inhibition of hyphal growth and reduction of spore germination, probably by interaction with pathogen receptors.²³ In this context, the presence of PR5K-related protein in the Rs23A secretome may mimic its plant counterpart and neutralize receptor binding on *R. solani*, thereby circumventing a portion of the plant defense response. GMC oxidoreductase and laccase, detected in the Rs23A secretome, may also function in counteracting the plant defense response. GMC oxidoreductase has been shown to reduce production of antifungal pathogen-induced metabolites while laccase has been shown to detoxify plant defense metabolites.²⁸

This investigation is the most comprehensive undertaking to date regarding proteomic profiles of cellular and excreted proteins of a *R. solani* AG 4 isolate. Our analysis has identified a number of proteins with possible roles in virulence by this soilborne necrotrophic plant pathogen. Future genomic, transcriptomic, and proteomic work with *R. solani* AG 4 isolate Rs23A, along with the complete genome sequencing and annotation of other *Rhizoctonia* genomes, will reveal more proteins with roles in pathogenicity allowing the generation of models regarding pathogenicity. Secretome studies with various inducers will identify effectors

triggering pathogenic success (i.e. pathogenic/virulence factors), stress response, and plant defense (i.e. avirulence factors) at the host-pathogen interface. This work will also reveal more proteins involved in mating relationships, morphogenesis, and ecology. The culmination of these studies will result in a more complete understanding of the molecular basis of pathogenesis by *R. solani* AG 4 and better tools for the detection and control of this economically important fungus.

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ASSOCIATED CONTENT

Supplementary Information

The Supporting Information is available free of charge via the internet at <http://pubs.acs.org>.

Supplementary Tables S1, S2, and Figures S1, S2 (PDF)

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Figure. 1. 2-D gel images of the *Rhizoctonia solani* AG 4 isolate Rs23A secretome when this fungus was grown on 112 mM glucose (medium #3; Panel A) or 14 mM glucose plus 0.5 g L⁻¹ citrus pectin (medium #4; Panel B) as the carbon source. First dimension resolution was carried out by isoelectric focusing at pH 4-7, while second dimension resolution was with 12% SDS-PAGE. Images are representative of 2 gels of these preparations run under these conditions.

Figure. 2. 2-D gel images of the *R. solani* AG 4 isolate Rs23A secretome when this fungus was grown on 14 mM glucose plus a soybean seedling contained within a dialysis bag (medium #1; Panel A) or 112 mM glucose plus a soybean seedling contained within a dialysis bag (medium #2; Panel B), or 112 mM glucose and no soybean seedling (medium #3; Panel C). First dimension resolution was carried out by isoelectric focusing at pH 4-7, while second dimension resolution was with 12% SDS-PAGE. Images are representative of 2 gels of these preparations run under these conditions.

Figure 3. Functional classification of secreted proteins of *Rhizoctonia solani* AG 4 isolate Rs23A at GO level 3 for biological process and molecular functions.

Figure 4. 2-D gel images of total intracellular proteins of *Rhizoctonia solani* AG 4 isolate Rs23A grown on medium #5. First dimension resolution was carried out by IEF at pH 4-7 (Panel A) and at pH 6-11 (Panel B), respectively. Second dimension resolution was done in 12% SDS-PAGE. The numbered protein spots were processed for MALDI-TOF-MS-MS analysis and protein identification.

Figure 5. Functional classification of intracellular proteins of *Rhizoctonia solani* AG 4 isolate Rs23A at the GO level 3 for biological process and molecular functions.

Figure 1.

Figure 2.

Figure 3.

Panel A

Panel B

Figure 4.

Figure 5.

TOC Graph